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Renal Excretion of 5,5-Dimethyl-2,4-oxazolidinedione (Product of Demethylation of Trimethadione).* (23540)

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The antiepileptic drug, trimethadione (Tridione), is converted by metabolic demethylation to 5,5 - dimethyl - 2,4 - oxazolidinedione ("DMO"). The conversion of trimethadione to DMO is nearly complete in the dog and in man(1). When trimethadione is administered in chronic schedules, DMO accumulates until the amount in the body is many times greater than the daily dose of trimethadione. Plasma concentrations of DMO as high as 1 g/l have been observed in patients receiving therapeutic doses of trimethadione. It appears likely that DMO plays an important role in the antiepileptic effect produced by administration of trimethadione. DMO is among the most persistent of all organic compounds that have been studied, the plasma concentration in man declining by only 5 to 12% per day after discontinuation of dosage. The extraordinary persistence of DMO is indicative of a very slow rate of renal excretion. An investigation of the mechanism of renal excretion of DMO is the subject of the present report.

Methods. Concentrations of DMO in plasma were determined by the ultraviolet spectrophotometric method of Butler(1). Because of interfering materials in urine this method is not applicable to urine. For the *determination of DMO in urine* the method was modified in the following way. To 1 ml of urine in a glass stoppered tube are added 25 ml of redistilled reagent grade absolute ethyl ether saturated with water and 4 ml of 5 M NaH_2PO_4. The tube is shaken and centrifuged. The ether phase is transferred by pipette to another tube containing 50 mg of Norit. After shaking and centrifugation 20 ml of the ether is transferred to another tube, where it is shaken with 5 ml of 0.05 M borate buffer, pH 9, previously saturated with ether. The tube is centrifuged and 2 ml por-

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† Public Health Service Research Fellow of the Natl. Inst. of Neurological Diseases.

tions of the lower phase are transferred to each of 2 tubes. To one is added 2 ml of the buffer of pH 9 and to the other 2 ml of 0.1 N HCl. Absorption measurements are made on these 2 preparations with a Beckman DU ultraviolet spectrophotometer, the blank for the first being the buffer and for the second a mixture of equal volumes of buffer and 0.1 N HCl. Absorbancies of both preparations are measured at 215 and at 220 mμ. The absorbancies at pH 9 are corrected by subtracting the corresponding absorbancies in acid solution. The concentration of DMO is calculated from the difference between the corrected absorbancy at 215 mμ and that at 220 mμ. By the procedure of partitioning between ether and buffers as described by Butler(1), the specificity of this method has been demonstrated. *Administration of drugs* to dogs. DMO was given intravenously in aqueous solution in a dose of 50 mg/kg at least an hour before a clearance determination. Diuresis with acid urine was produced by administration of 30 ml of water per kg by stomach tube, 130 mg of meralluride sodium intravenously, or 50 to 100 ml of 0.95 M Na_2SO_4 intravenously. Excretion of alkaline urine was brought about by intravenous administration of 50 to 100 ml of 8% (w/v) $NaHCO_3$. *Collection of urine* from dogs was by catheter. Urine for pH determination was kept in glass stoppered flasks and the pH measured within 10 min. of the time of collection. *Determination of pH, measurement of protein binding, and measurement of creatinine clearances in dogs* were performed by the methods used in an earlier study of phenobarbital(2).

Results. Protein binding. In 4 g/100 ml solutions of bovine serum albumin in buffers of pH values of 6.0 to 8.5, no binding of DMO to the protein could be detected.

Extent of excretion. A 10 kg dog was given an intravenous dose of 0.5 g of DMO and the urine collected from an indwelling catheter. Excretion of DMO was accelerated by administration of several intravenous infusions of a $NaHCO_3$ solution. This treatment resulted in the plasma concentration falling to a negligible level in 2 days. Normally about 2 weeks would have been required for this to occur. The DMO as determined in the urine excreted during these 2 days accounted for 99% of the administered dose. DMO does not undergo metabolic change to any significant extent in the dog. Renal excretion is the only process of any importance in the elimination of the compound.

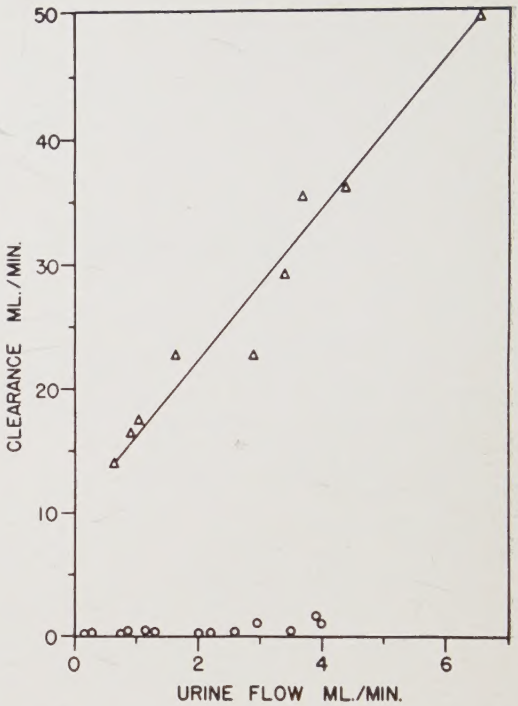

FIG. 1. Renal clearances of DMO in a dog. Values designated by circles are from experiments in which diuresis was induced by oral water, intrav. meralluride sodium, or intrav. Na_2SO_4, and in which the urinary pH was below 7.0. Values designated by triangles are from experiments in which $NaHCO_3$ was given intrav. and in which the urinary pH was 7.8 to 8.0.

Effect of urine flow and pH on excretion of DMO. Several measurements of the renal clearance of DMO were made in the same dog at different times under different conditions. The results are shown in Fig. 1. When there was no diuresis or when diuresis was brought about by water, meralluride, or Na_2SO_4, the pH of the urine was below 7 and the clearances of DMO were very low even at the highest flow rates. Urine/plasma concentration ratios as low as 0.1 were found when the urine was acid. After administration of $NaHCO_3$, the urinary pH was 7.8 to 8.0. The clearances of DMO were much higher than when the

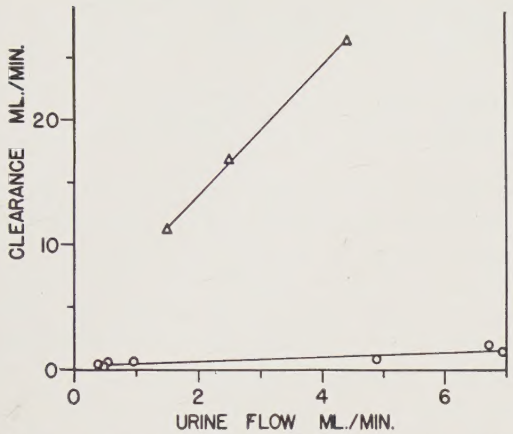

FIG. 2. Renal clearances of DMO in a man. Values designated by circles are from an exp. in which diuresis was induced by administration of 1 l of water by mouth. Urinary pH was 5.6 to 6.4. Values designated by triangles are from an exp. in which the same man was given 15 g of $NaHCO_3$ intrav. Urinary pH was 8.0.

urine was acid, and they increased with increasing rate of flow. Clearances with alkaline urine at high flow rates were as much as 250 times those with acid urine. However, the highest clearances did not equal the creatinine clearance.

Fig. 2 shows DMO clearances in a man who had been receiving 800 mg of DMO daily for several weeks. As in the dog, the clearances were very low when the urine was acid, even when the flow rate was high. The U/P ratio varied from 0.7 at low flow rates to 0.2 at high flow rates. When the urine was alkaline, the clearance was greatly increased and varied to an important extent with flow rate.

Discussion. There are a number of examples of organic acids that are excreted more rapidly in alkaline than in acid urine and of bases that are excreted more rapidly in acid than in alkaline urine (2,3). This effect has been explained in terms of a renal tubular epithelium permeable to the lipid-soluble undissociated forms of acids and bases but impermeable to the ionic forms. DMO is an acid with a pK′ value of 6.13 at 37°C and ionic strength of 0.16. The influence of urinary pH on the excretion of an acid with this value of pK′ would theoretically be predicted to be important. If an acid with pK′ = 6.13 were fully equilibrated between plasma with pH 7.4 and urine with pH 8.0, the two phases being separated by a membrane permeable only to the undissociated form, the U/P concentration ratio for the acid would be 3.8. If plasma of the same pH were equilibrated with urine of pH 6.0, the U/P ratio would be 0.09. Equilibration is never actually complete, and the amount of DMO reabsorbed is less than that predicted. The observed values of U/P are accordingly higher than the theoretical values both in acid and alkaline urine. The effect of urinary pH on the excretion of DMO can thus be explained on the assumption that the drug is filtered and then reabsorbed by a process of passive diffusion, the tubular epithelium being impermeable to the ionic form. The observed U/P ratios lower than unity in acid urine can be accounted for without the assumption of any "active transport" system.

During the greater part of the day the urine of a man on an ordinary diet is at pH values so low that the U/P ratio for DMO is at or below unity. The very extensive tubular reabsorption with acid urine together with the insusceptibility to metabolic attack account for the remarkable persistence of DMO.

Summary. A method is described for determination of 5,5-dimethyl-2,4-oxazolidinedione (DMO) in urine. DMO is not bound to serum albumin. In the dog DMO is excreted completely unchanged in the urine. In the dog and in man the renal clearance of DMO is much higher in alkaline urine than in acid urine. This is explained on the assumption that the compound is reabsorbed in the renal tubule by a process of passive diffusion, the tubular epithelium being permeable to the undissociated form and impermeable to the ionic form.

We are indebted to Dorothy Johnson and Anne Kulcsar for technical assistance.

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Effect of Synthetic Vasopressin on Release of Adrenocorticotrophin in Rats with Hypothalamic Lesions.* (23541)

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The hypothesis that the antidiuretic hormone (ADH) regulates the release of adrenocorticotrophin (ACTH) from the adenohypophysis is supported by the following evidence: i) ADH and ACTH are released under similar circumstances(1). ii) A defect in ACTH release is correlated with diabetes insipidus in rats with hypothalamic lesions(2). iii) Pitressin, a commercial extract containing the pressor-antidiuretic activity of the posterior lobe, releases ACTH in rats with lesions which block the release of ACTH to a variety of non-specific stimuli(2,3). iv) There is a correlation between pressor and ACTH-releasing activity of posterior lobe extracts, suggesting that ACTH-releasing activity may be a property of the pressor-antidiuretic hormone(3). Extracts of the posterior lobe having pressor activity appear to release ACTH from the pituitary also *in vitro,* but a similar activity has been obtained from extracts possessing little or no pressor activity(4,5). Therefore, in the present experiments the ACTH-releasing activity of synthetic vasopressin† has been evaluated as a further test of the hypothesis that the neurohumor stimulating the release of ACTH is vasopressin itself.

In these experiments rats with acute hypothalamic lesions were used as assay animals since it has been shown that these rats do not respond to a variety of non-specific stimuli while retaining their response to ACTH(1). Hence, it has been assumed that these lesions block the pituitary-adrenal stress response at the hypothalamic level and that the animals should respond to the presumed neurohumor which evokes the secretion of ACTH.

Methods. Hypothalamic lesions were placed in the median eminence of the tuber cinereum of male Wistar rats (270-330 g) as previously described(3). Those animals with water intakes in excess of 110 ml in the first 24 hours after the operation were used for assay 24 hours later (*i.e.* 48 hr post-op), providing they appeared to be in good condition. Adrenal ascorbic acid depletion was used as the index of ACTH release in all experiments. The left adrenal was removed under ether anesthesia to obtain a control value for adrenal ascorbic acid. One hour after unilateral adrenalectomy or injection of a test drug, the second gland was removed for analysis(3).

Results. The rats with hypothalamic lesions showed no response to unilateral adrenalectomy alone (Table I). In contrast, both Pitressin and synthetic vasopressin elicited a significant ascorbic acid depletion (Table I, Fig. 1). The responses to the 2 drugs were indistinguishable when dosage was expressed in pressor units. The minimal effective dose of both preparations was of the order of 0.1 International Unit (U). On assay at varying dosages, Pitressin exhibited a potency 83% that of synthetic vasopressin, considering the synthetic material the standard at 100%. The 95% confidence limits of the assay were 32-219%.‡

Thus, these results clearly show that synthetic vasopressin can release ACTH in this experimental situation. It seems highly likely, then, that the vasopressin molecule itself possesses intrinsic activity in the release of ACTH. The activity of commercial Pitressin in releasing ACTH in these experiments can be entirely accounted for by its content of vasopressin. Therefore, there seems no need to postulate that a contaminant with significant ACTH releasing action is present in posterior lobe extracts in the doses employed. Ten U of Pitocin, a commercial preparation of

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† Kindly supplied by Dr. V. du Vigneaud, Dept. of Biochemistry, Cornell Univ. College of Med.

‡ Calculated by the method of Irwin (see Pugsley, L. I., *Endocrinol.,* 1946, v39, 161).

TABLE I. Comparison of ACTH-Releasing Activity of Pitressin with That of Synthetic Lysine-Vasopressin in Rats with Acute Hypothalamic Lesions.

Treatment	No. of rats	Adrenal ascorbic acid depletion (mg/100 g adrenal)	
		Mean ± S.E.	Individual responses
1. Control*	6	−6 ± 8	−2, −4, 12, 10, −42, −10
2. Pitressin			
5 U†	3	158	116, 199, 160
.5 U	6	96 ± 19	108, 141, 147, 18, 80, 80
.25 U	5	74 ± 14	104, 31, 86, 49, 98
.1 U	4	36 ± 19	13, 15, 23, 94
3. Lysine-vasopressin			
1.0 U	7	125 ± 30	112, 258, 123, 54, 130, 67, 161
.5 U	6	77 ± 21	27, 55, 148, 131, 72, 28
.25 U	6	77 ± 19	147, 20, 83, 34, 102, 79
.12 U	7	55 ± 21	14, −6, 30, 94, 89, 140, 27
.062 U	5	31 ± 7	37, 17, 27, 17, 55
4. Pitocin			
10 U	7	44 ± 18	2, 64, 50, −13, 10, 90, 115

* Unilateral adrenalectomy only; all other animals were inj. with the drug to be tested immediately after unilateral adrenalectomy.

† All inj. given intrav. during a period of 3 min.

the oxytocic hormone, evoked no significant ACTH release ($P<0.1>0.05$). Since this dose is approximately 100 times the minimal effective dose of vasopressin, it appears that oxytocin plays little or no role in the release of ACTH.

Because of the failure of these rats with lesions to respond to a variety of non-specific stimuli, it appears reasonable to assume that the action of vasopressin in these experiments is directly on the adenohypophysis. The large dose of vasopressin required to release ACTH in these experiments suggests that if vasopressin is to act as the ACTH-releasing neurohumor, it would need to be released into the hypophyseal portal vessels so as to reach the adenohypophysis in a relatively high concentration.

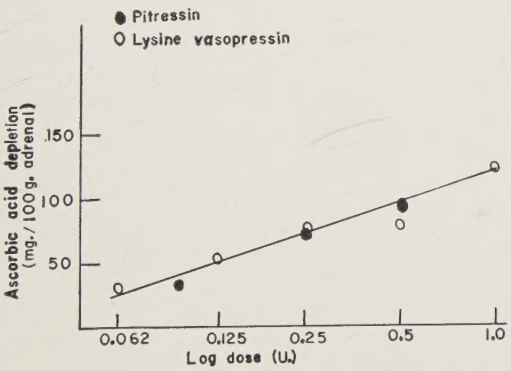

FIG. 1. Activity of posterior lobe extracts.

Conclusions. Commercial and synthetic vasopressin are equipotent in producing ascorbic acid depletion in rats with those hypothalamic lesions which prevent the adrenal response to certain non-specific stimuli. The results are interpreted to mean that vasopressin can evoke ACTH release in these animals and that the ACTH-releasing activity of neurohypophyseal extracts, when tested *in vivo*, is accounted for by their content of vasopressin. The results support the hypothesis that vasopressin (ADH) is the neurohumor responsible for ACTH release.

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A Simple Method for Preparation of Highly Purified Vasopressin.* (23542)

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In the course of a series of experiments toward the characterization in the hypothalamus of the mediator of ACTH-release(1) it became imperative to study the possible role of vasopressin in this phenomenon. This required that milligram quantities of highly purified vasopressin be available for biological studies and also for physico-chemical comparison with the ACTH-releasing substance which, on the basis of the extraction procedure and other criteria, appeared to be related in some ways to the pressor principle(1). Highly purified arginine-vasopressin (AVP) and lysine-vasopressin (LVP) have been prepared by methods which all require days or weeks (2,3,4) for completion and usually large quantities of starting material. The method proposed here permits the separation of highly purified vasopressin in a few hours and with yields high enough to allow starting with reasonably small quantities of crude material. Successful chromatography of vasopressin has been described using Amberlite IRC-50 ion exchange resin with phosphate(5) or ammonium acetate(6) buffers. Condliffe has used partition chromatography(7). All these procedures suffer from the disadvantage of extremely low flow rates and capacity. In the studies described here, high capacity and flow rate have been obtained using a cellulose ion exchanger.

Methods. A carboxymethyl cellulose ion exchanger prepared according to the method of Peterson and Sober(8) and possessing a capacity of 0.65 meq/g was used for the present studies. Starting material for the chromatography was obtained by fractionation of Proto-pituitrin (Parke-Davis) by the method of Kamm *et al.*(9). For the LVP a fraction with a potency of 22 pressor units/mg (equivalent to combined fractions "e" and "f" in the original Kamm procedure) was used, while a similar fraction from beef Proto-pituitrin having a potency of 35 u/mg was used for the AVP. Chromatography was carried out in glass tubes approximately 1 cm in diameter and with a height of 25 cm or 60 cm. Material to be chromatographed was dissolved in 0.02 M ammonium acetate buffer, pH 6.0, and applied to the column previously equilibrated with this same buffer. Development of the column was then carried out using a gradient through a 250 ml mixing chamber(10) to either a 0.1 M or a 0.2 M ammonium acetate buffer, pH 7.0. Protein was measured by U.V. absorption at 275 mμ and the Folin-Lowry reaction(11). After the vasopressin peak was located by bioassay of aliquots of the effluent the appropriate fractions were pooled and lyophylized. Using an all-glass lyophilizer and a vacuum pump operating at 0.1 mm of Hg, all detectable ammonium acetate was removed after the material was redissolved in water and lyophilized one more time at temperatures not exceeding 25°C. Material dried in this manner was then used for the final determination of specific activity. Assays for pressor activity were carried out using the rat blood pressure method of Dekanski(12). For determinations of the activity contained in aliquots of the effluents, bracketed assays were used. To measure the specific activity of the final lyophilized products, four-point-assays in duplicate were run and calculated according to Bliss(13). All assays were done using USP posterior pituitary Reference Standard with a stated potency of 0.4 u/mg (recently revised downward from 0.47 u/mg).

Results. Fig. 1 shows the chromatogram of 750 mg of partially purified lysine vasopressin (22 u/mg) on a 25 cm colum containing 2.5 g of exchanger, with hold-up volume of 18 ml and operated at a flow rate of

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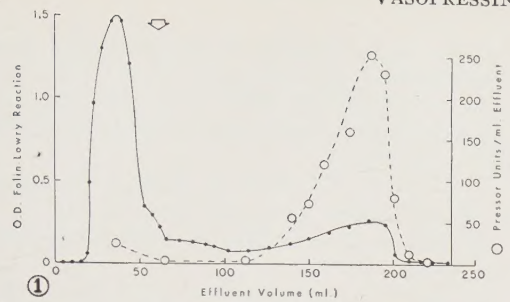

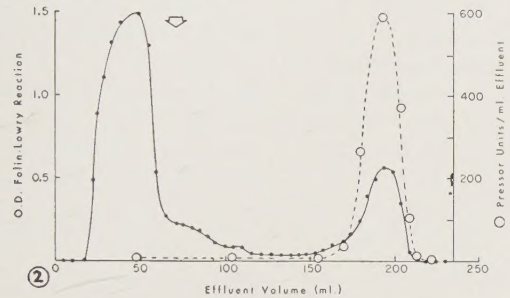

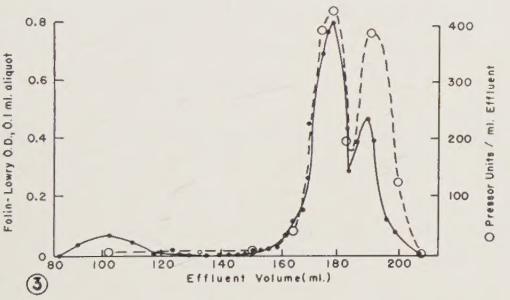

FIG. 1. Chromatography of lysine vasopressin on carboxymethyl cellulose. 750 mg with potency of 22 u/mg applied to the column (1 × 25 cm) in 0.02 M ammonium acetate buffer, pH 6. Gradient to 0.1 M ammonium acetate buffer, pH 7, through 250 ml mixing chamber was begun at the point marked by the arrow. ●—Folin-Lowry color, 0.05 ml aliquots. ○—Pressor activity of effluent.

FIG. 2. Chromatography of arginine vasopressin on carboxymethyl cellulose. 500 mg with a potency of 35 u/mg applied to the column (1 × 25 cm) in 0.02 M ammonium acetate buffer, pH 6. Gradient to 0.2 M ammonium acetate buffer, pH 7, through 250 ml mixing chamber was begun at the point marked by the arrow. ●—Folin-Lowry color, 0.1 ml aliquots. ○—Pressor activity of effluent.

FIG. 3. Chromatography of arginine and lysine vasopressin mixture on carboxymethyl cellulose. 15 mg lysine vasopressin (250 u/mg) and 10 mg arginine vasopressin applied to the column (1 × 60 cm) in 10 ml pH 6, 0.02 M ammonium acetate buffer. Gradient to pH 7, 0.2 M ammonium acetate buffer through 250 ml mixing chamber started after 12 ml of effluent. Small peak at 100 ml effluent vol represents an impurity present in this particular lysine vasopressin sample. ●—Folin-Lowry color, 0.1 ml aliquot. ○—Pressor activity of effluent.

approximately 30 ml per hour. 60 mg of vasopressin were isolated from the pressor area (150-205 ml effluent) with a specific activity of 260 u/mg. Hydrolysis of this material with 6N HCl showed all the amino acids of LVP to be present(3,14) plus traces of alanine and histidine as revealed by paper chromatography of 1 mg of the hydrolyzate.

Rechromatography of this material on a longer column gave a product with essentially the same specific activity, but containing only the amino acids of LVP. The potency and purity of this material thus appear as good as that reported for LVP obtained by other methods(3).

Fig. 2 shows a similar chromatogram of partially purified AVP (500 mg, 35 u/mg) as with the LVP, except for the elution gradient which was to a 0.2 M ammonium acetate buffer, pH 7.0. The pressor material (172-212 ml effluent yielded 40 mg after lyophilization) assayed 380 u/mg and contained only the 8 amino acids of AVP(2). This material thus has a lower potency than the most potent material obtained by Popenoe *et al.*(15), although subsequent preparations by the Cornell group have exhibited lower potencies, yet contained only the 8 amino acids of AVP(7, 16). Thus we feel that this represents a reasonably good purification of AVP.

From the difference in the elution gradient employed for LVP (Fig. 1) and AVP (Fig. 2) it was anticipated that the two hormones could be resolved by chromatography on carboxymethyl cellulose. Accordingly, 15 mg of LVP (250 u/mg) and 10 mg of AVP (380 u/mg) were mixed and applied to a column 1 x 60 cm with 40 ml hold-up volume and 5 g exchanger in pH 6.0, 0.02 M ammonium acetate buffer and a gradient to 0.2 M ammonium acetate buffer pH 7.0 used to develop the column. Under these conditions it can be seen (Fig. 3) that LVP and AVP are resolved. Only the second pressor peak gave a positive Sakaguchi reaction. Thus the AVP showed a greater affinity for the column, as would be expected from its higher isoelectric point, 10.9(5,17), compared to 10.0 for LVP.† The higher ratio of activity to Folin-Lowry O.D. for the AVP peak in Fig. 3, reflects the greater

† Unpublished results, D. N. Ward.

potency per unit weight for AVP(3,15).

Summary. Both arginine and lysine vasopressin have been obtained in a high degree of purity by means of chromatography on carboxymethyl cellulose using ammonium acetate as a volatile buffer and differential elution gradients. Conditions for the resolution of LVP and AVP by chromatography on this same exchanger are described. By combining efficient recovery of activity, high flow rates for the chromatography, and rapid removal of buffers by lyophilization, a simple means of purifying vasopressin is proposed.

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Effect of a Histamine Releaser (48/80) upon Development of Walker Tumor.* (23543)

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The possibility of depleting a tissue of its histamine content by repeated injection of compound 48/80(1) suggests a method by which to evaluate the role of histamine in tissue injury. This method was first used in our studies on inflammation. In rats treated with compound 48/80 or various histamine releasers the tissue responsiveness is either enhanced or decreased, depending upon the nature of the irritant utilized(2).

In the course of our studies on the relationship between inflammation and cancer we thought it would be interesting to investigate the influence of compound 48/80 upon development of a transplanted tumor. The present experiments suggest that this histamine releaser, especially when given as pretreatment, is particularly effective in enhancing the development rate of Walker tumor.

Materials and methods. Three experimental series were made with female Sprague-Dawley rats of an average initial body-weight of 60 g (range 58 to 64 g). It was planned to determine whether the development of the tumor would be influenced by 48/80‡ administered before transplantation (Exp. 1), during tumor-growth (Exp. 2), and after the tumor is well developed (Exp. 3). In each experiment the compound was injected subcutaneously, twice daily, for 8 days; doses

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† The author wishes to acknowledge technical assistance of Miss Alice Parisi and appreciation to Jacques Letarte for statistical analyses.

‡ The 48/80 used was kindly supplied by Dr. Edwin J. de Beer of Wellcome Research Laboratories, Tuckahoe, N. Y.

TABLE I. Mode of Treatment with 48/80.

Day	Dose of 48/80 (μg) A.M.	P.M.
1		200
2	200	"
3	"	300
4	300	"
5	400	400
6	"	500
7	500	"
8	"	"

were increased progressively as outlined in Table I. Throughout the experiments, the rats were maintained on "Purina Fox Chow" and received additionally some "Pablum." This dietary supplement was given to increase the animals' appetite, and, thereby, to lessen the possibility of gastric ulcers which may occur during long term 48/80 administration. A suspension of Walker Carcinoma 256 was prepared by crushing only the living tissue of an 8-day-old tumor in a glass homogenizer, using 1 g of tumor to 5 ml of physiological saline. The animals were lightly anesthetized with ether and each received 0.2 ml of the suspension underneath the skin of the central lumbar region. The transplantation was carried out under sterile conditions. To ascertain that distribution was as even as possible, cells from a single donor were used and the animals of all experiments were injected at the same time. The rats were sacrificed with chloroform on the 15th day in Exp. 1 and 3 and on the 8th day in Exp. 2. The tumor was dissected and fixed in Susa solution for subsequent weighing and histologic studies.

Results. Table II summarizes the results of the 3 experiments. The absolute tumor weight values reveal a definite growth promoting effect of 48/80 in all the experimental arrangements used. Gross inspection of animals in Exp. 1 showed differences in tumor sizes, already detectable on the fourth day following the transplantation. In Exp. 2 and 3 appraisal was more difficult because of the tendency of the tumor to form fluid underneath the skin, thus making its delimitation somewhat irregular. Once the neoplastic tissue had been carefully dissected and weighed, the sizes became more uniform.

For statistical analysis the tumor weight has been expressed per 100 g of body weight. On this basis, only pretreatment with 48/80 (Exp. 1) seems effective in increasing the weight of the tumor. The number of animals used throughout the experimental series was small, and it is thought that had a larger number been used, significant results may also have been obtained in Exp. 2 and 3.

At autopsy, no important organ change was found except a slight adrenal hypertrophy which was uniform in all groups. Upon histologic examination, tumors of 48/80 treated rats were essentially comparable to those of untreated animals. Active neoplastic growth and infiltration of the skin with tumor cells were observed; in no case was there any appreciable degree of necrosis that might have accounted for the differences in tumor weight.

Discussion. Previous studies(3,4) in which 48/80 was given at the time of transplantation, did not reveal any appreciable effect upon Walker tumor. Pretreatment, on the other hand, shows a definite influence on the course of the tumor growth.

TABLE II. Effect of Compound 48/80 upon Development of Walker Tumor.

Exp. No.	Duration (days)	48/80 treatment relative to transplantation	Tumor absolute (mg)	Tumor/100 g body wt (mg)	Body gain (mg)	Mortality
1	15	—	2607 ± 420	1973 ± 308 ($P < .02$)	74 ± 3	0/8
		Before	4450 ± 588	3812 ± 593	60 ± 4	1/8
2	8	—	2498 ± 545	2679 ± 603 ($P < .1$)	33 ± 12	0/8
		Simultaneously	3277 ± 437	4419 ± 714	15 ± 3	1/8
3	15	—	5524 ± 1593	4522 ± 970 ($P < .4$)	72 ± 7	0/8
		After	10183 ± 1710	6017 ± 1363	72 ± 5	1/8

Histamine has been reported to exert an inhibitory effect on the growth of transplanted tumors(5). The present results are in accord with this observation.

Since tumor growth was more favorable in histamine depleted animals, it is tempting to assume that tissue reactivity or inflammatory potential regulates tumorigenesis. In order to determine to what extent this hypothesis could further be verified, experiments are now underway to investigate whether compound 48/80 might influence the rate of tumor induction in rats.

Summary. The growth of Walker Tumor 256 is increased in rats receiving compound 48/80 in progressive dosage. This is significant only in animals pretreated with the histamine releaser.

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A DNA-Reacting Factor in Serum of a Patient with Lupus Erythematosus Diffusus.* (23544)

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The question whether DNA can elicit specific antibodies in experimental animals is still controversial, although some positive evidence has been presented(1). Our own experiments have been inconclusive(2). Because of the possibility that DNA might behave as an hapten, it was nevertheless decided to test whether DNA preparations would react against human pathological sera. Our attention was mainly directed towards Lupus Erythematosus for: 1) the nucleolysis and subsequent nucleophagocytosis typical of this condition, liberate and possibly make accessible to immunological processes an abnormally large amount of nuclear material; 2) these patients are exceptionally apt to produce auto and iso-antibodies; 3) the immunological nature of the L.E. phenomenon is supported by some well established observations.

The following sera have been tested against DNA: a) 9 from Lupus Erythematosus diffusus, b) 60 from normals, c) 15 from luetic patients, d) 30 from miscellaneous diseases showing high level of gamma globulins, e) 7 from collagenous diseases (1 Liebman-Sachs endocarditis, 3 cutaneous Lupus Erythematosus, 1 Schoenlein-Henoch, 1 macroglobulinaemia). A definite positive reaction on complement fixation and on precipitin test was observed only with the serum of a patient with acute, untreated, Lupus Erythematosus diffusus (serum E-2); all other sera did not react. A short account of the observations herein presented has been previously published(3).

Materials and methods. DNA was obtained from i) normal human leucocytes, ii) leucocytes from human lymphatic leukemia, iii) leucocytes from human myeloid leukemia, iv) calf thymus, v) rabbit spleen. DNA was extracted according to the Kay, Simmons, Dounce technic as modified by Chargaff(4), using 10^{-3} M NaCl as the solvent wherever water was prescribed (Cavalieri *et al.,* 1956). Moreover the crude sodium nucleate was only washed with 95% ethanol. The fibrous DNA was stored at –20°C. The chemical analysis *i.e.* % H_2O, N, P(5), the u.v. absorp-

* We wish to express our gratitude to Dr. R. Pezzi for the supply of the treponemic extract, to Dr. A. Zamboni for preparation of pure crystallized enzymes, to Prof. A. Fanconi, Dr. A. Marmont, Dr. P. Hollaender for the supply of some L. E. sera and to Mr. M. Turri for technical assistance.

tion data at various pH, the average molecular weight as determined by light scattering (6) and the sedimentation experiments before and after chymotrypsin showed the high degree of purity of these undenaturated DNAs. As a control the following antigens were tested against E-2 serum: RNA from yeast and from human leucocytes; purified Vi and O polysaccharides from *S. typhi;* purified H blood group substance from human meconium; polyvinylpyrrolidone; dextran; cardiolipine; an aqueous extract from *Treponema pallidum,* Reiter's non-pathogenic strain. The sodium salt of the DNA used for the immunological test was dissolved in 0.14 M NaCl by standing overnight at 4°C (concentration 500 g/ml). Further dilutions in the range of 100-0.5 μg/ml were prepared immediately before use; none of our DNA preparations up to a concentration of 100 μg/ml showed any anticomplementary activity. The anticomplementary activity of the E-2 serum was compensated for by titrating the complement in presence of 1 volume of diluted (1:4) inactivated serum. Equal amounts (0.10 ml) of: 1) guinea pig complement containing two 50% units, 2) diluted (1:4) E-2 serum, 3) various dilutions of antigen, were mixed; 0.20 ml of veronal buffered saline were added and the mixture left overnight at 4°C. The following morning 1 ml of sensitized red cells was added and the hemolysis read after 45′ incubation at 37°C. A full range of serum and antigen controls was set up in all tests. A positive reaction was scored when hemolysis was reduced from 90% to 30% or less. The precipitin tests were carried out in capillary tubes (bore 2 mm) stratifying the antigen at various concentrations over the serum usually diluted 1:4 and clarified by removal of lipids. A reaction was judged positive when a sharp opaque disk appeared at the interface within 2 hours; in general the reaction was visible after 15′ and reached its maximum in 45′.

Results. A) The E-2 serum gave a positive reaction both as complement fixation and as ring precipitation with all DNA preparations tested. Within the sensitivity of the methods no differences in reactivity among DNAs

TABLE I. Schematic Representation of Serological Reactions (Precipitin Ring Test and Complement Fixation) Given by Different Sera against Pertinent Materials. Note disappearance of reactivity after treatment of DNAs and Treponemic extract with DNAase.

Sera	DNAs (1-100 μg/ml)			Treponemic extract (1:5-1:20)			Cardiolipine	Other macromolecular RNA antigens (1-100 μg/ml)	
	Untreated	+ DNAase	+ chymotrypsin	Untreated	+ DNAase	+ chymotrypsin			
Lupus Erythematosus (Patient E-2)	++	0	++	+	0	++	0	0	0
Lues	0	0	0	++	++	±	++	0	
Normals, 9 other L.E. sera and miscellaneous diseases	0	0	0	0	0	0	0	0	

++ = strongly positive; + = positive; ± = weakly positive; 0 = negative.

from different sources were detected, all preparations reacting positively up to a concentration of about 1 μg/ml. Among the other above listed materials only the treponemic extract behaved like DNA.

B) The finding that E-2 serum reacted with the treponemic extract, but did not with cardiolipin and gave a negative treponemic immobilization test led us to investigate if nucleic acids were present in the spirochetal material; using the Ceriotti method it was possible to show the presence of DNA (10-20 μg/ml).

C) Absorption of E-2 serum for one week at 4°C with an excess of any one of the 5 DNA preparations and of the treponemic extract, neutralized all precipitating reactivity, while absorptions with unrelated materials, including RNA, were ineffective.

D) One DNA preparation and the treponemic extract were again tested after treatment under appropriate conditions with crystallized DNAase and Chymotrypsin; the results are particularly significant (Table I) and strongly suggest that only DNA was involved in the reactivity of the E-2 serum.

E) The reacting factor was still active after 1 hour heating at 56°C. The precipitate from the E-2 serum at ½ saturation of $(NH_4)_2SO_4$ reacted strongly on ring test with DNA, but could not be used for the complement fixation because of a very high anticomplementary activity. Preliminary experiments of recovery of the factor from the different fractions of the serum, isolated by means of zone electrophoresis, have been up to now unsuccessful.

Conclusions. While further investigations are in progress, the following conclusions may be provisionally drawn: 1) in serum of a patient with acute Lupus Erythematosus diffusus a factor has been found specifically reacting with purified DNA; 2) this factor does not discriminate between DNAs from different sources and species; 3) under usual criteria it seems to behave like an antibody; 4) the positive reaction of this peculiar serum against a treponemic extract appears to be due to the presence of desoxypentose nucleic acids in the extract. 5) It has not been possible to test whether the serum of this patient, which gave a strongly positive L. E. phenomenon, would retain this ability after absorption with DNA; hence it is impossible now to decide how closely this factor is related to the Hargrave Haserick's globulin. Because no reactivity against DNA was demonstrated in 8 other patients with a positive L.E. phenomenon, the DNA reacting factor of this peculiar serum might be regarded as an occasional expression of the disease, related to the wellknown auto-immune hyperreactivity of the L.E. patients. If the factor is demonstrated not to be an antibody the alternative explanation could be of a material of nuclear origin still able to form a complex with DNA.

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Complement Fixation with Cell Nuclei and DNA in Lupus Erythematosus. (23545)

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Evidence has been presented(1) that the lupus erythematosus (L.E.) serum factor reacted specifically with nuclei and nucleoprotein. It could be readily removed from serum by absorption on nuclei or nucleoprotein and active material could be recovered by elution. Localization on the nuclei was demonstrated by the fluorescent antibody technic as well as by the ability of such nuclei to be phagocytized to form L.E. cells. Similar results were obtained with isolated nucleoprotein. The possibility was raised that the L.E. factor was an antibody; it had the physical properties of a γ-globulin and appeared to react with antiserum to normal γ-globulin. In order to explore this concept further, experiments with complement fixation have been carried out to see if complement is removed in the reaction between the L.E. factor and nuclei. Desoxyribosenucleic acid (DNA) as a major constituent of nuclei also has been studied by this technic. It was felt that a complement fixation reaction specific for this disease or for the L.E. factor might have advantages over the L.E. test clinically as well as furnishing information regarding possible antinuclear antibodies.

Materials and methods. Sera: All sera were stored at 4°C and inactivated in a waterbath at 56°C for 30 minutes prior to use in complement fixation. *Preparation of nuclei*: Calf thymus nuclei were prepared by the method of Mirsky *et al.*(2). The human monocytes were obtained from a patient with acute monocytic leukemia. The cells were suspended in a solution of 0.25 M sucrose and 0.003 M $CaCl_2$, disrupted in a Potter homogenizer, and the nuclei separated by centrifugation. The rabbit leukocyte nuclei were prepared by a similar method from glycogen-induced peritoneal exudates. All nuclei were lyophilized and stored in a desiccator at 4°C. The lyophilized nuclei were suspended in buffer immediately prior to use with gentle mixing. None of the nuclear preparations was anticomplementary in the concentrations used (1 or 2 mg/ml) and suspensions of calf thymocyte nuclei were not anticomplementary even in a concentration of 10 mg/ml. *DNA*: Calf thymus DNA was prepared by the Sevag method and lyophilized.† It was dissolved in a small volume of distilled water with stirring and made to final volume with buffer. Powdered salmon sperm DNA was obtained from the California Foundation for Biochemical Research and was prepared for use similarly. Calf thymus DNA was strongly anti-complementary in concentrations of 500 γ/ml or higher, but was not at 100 γ/ml or lower. It was employed in complement fixation in a concentration of 5 to 100 γ/ml. These concentrations of DNA had no effect on complement titrations. Furthermore, complement fixation was stronger with concentrations of DNA of 5-20 γ/ml than with higher concentrations. *Complement fixation test*: This was performed after the method of Casals and Palacios(3). All dilutions were made in veronal saline buffer containing Ca and Mg ions, pH 7.4. Fresh guinea pig serum stored at –20°C was used as complement. It was titrated prior to use in each test, and was diluted so that 0.5 ml contained 2 units. The test was carried out as follows: 0.25 ml of serum serially diluted, 0.5 ml of complement containing 2 units, and 0.25 ml of nuclei suspension or DNA solution were mixed and incubated in a waterbath at 37°C for 30 minutes or at 4°C overnight. One-half ml of sensitized cells was then added and a second incubation at 37°C for 30 minutes carried out. Serum, antigen, complement and cell controls were included in each test. Fixation of complement was read as complete if there was no

† Obtained through the courtesy of Dr. Alfred Mirsky, Dr. Maclyn McCarty and Dr. Lewis Wannamaker.

TABLE I. Complement Fixation with Calf Thymus Nuclei.*

Serum	L.E. cell formation	Serum dilution 1	2	4	8	16	32	64	128	256	512
Syst. lupus eryth. (Fr.)	Strong	4	4	4	4	4	4	4	4	3	0
Idem (St.)	"	4	4	4	4	4	3	0	0	0	0
" (Ne.)	"	4	4	4	4	4	4	4	±	0	0
" (Ga.)	Medium	4	4	4	4	4	0	0	0	0	0
" (Hi.)	"	4	4	4	4	1	0	0	0	0	0
" (Ro.)	"	4	4	4	4	2	0	0	0	0	0
" (Sa.)	Weak‡	4	4	1	0	0	0	0	0	0	0
" (Ma.)	"	2	0	0	0	0	0	0	0	0	0
" (Ya.)	"	0	0	0	0	0	0	0	0	0	0
Misc. hyperglobulinemias	(9)	†	0	0	0	0	0	0	0	0	0
Rheumatoid arthritis	(21)	§	0	0	0	0	0	0	0	0	0
Miscellaneous diseases	(16)	†	0	0	0	0	0	0	0	0	0
Normal sera	(15)	†	0	0	0	0	0	0	0	0	0

* 2 mg/ml.

† A few of these sera were anticomplementary or showed slight fixation in the first 1 or 2 tubes, not considered significant.

‡ Those patients listed as "weak" gave distinctly positive preparations by the fresh heparin-bead method but weak to negative preparations by the Snapper method on stored sera.

§ Three of these sera had very high titers for sensitized sheep cell agglutinins. They were strongly anticomplementary. Parallel serum controls demonstrated that "fixation" in the first 2-3 tubes was due to the anticomplementary effect of the serum alone. The remaining 18 sera were negative throughout.

hemolysis (designated in Tables by 4), and as negative if there was complete hemolysis (designated in Tables by 0), with gradations of 3, 2, 1 and ±. A few sera were anticomplementary. In these instances parallel serum dilution controls to which no "antigen" was added were run for comparison, or the antigen dilution method employing a dilution of serum which was not anticomplementary was employed. *Absorption with nuclei and DNA*: Rabbit leukocyte or calf thymus nuclei were suspended in serum in a final concentration of 10 mg/ml, incubated in a waterbath at 37°C for 30 minutes, centrifuged at 2,000 rpm for 20 minutes and the supernate removed for testing. Lyophilized calf thymus DNA was added to serum in a final concentration of 1 mg/ml, incubated overnight at 37°C, centrifuged at 10,000 rpm for 2 hours, and the supernate removed for testing. In these experiments the L.E. test was done by the Snapper method.

Results. Experiments with cell nuclei: Positive complement fixation occurred with nuclei in 22 of 30 lupus sera. Results with representative lupus sera and a variety of other sera are presented in Table I. All lupus sera which were strong L.E. cell inducers showed complement fixation with nuclei at a dilution of 1:32 or greater. Lupus sera which were weak or negative L.E. cell inducers fixed complement in lower titer or failed to fix with calf thymus nuclei. The latter sera were usually from patients in clinical remission. Fixation was observed with calf thymus, human monocyte, beef liver and rabbit leukocyte nuclei. Most of the studies were carried out with calf thymus nuclei because of their availability.

The specificity of the reaction was tested on a limited scale by using sera from normal persons and from patients with other diseases characterized by hyperglobulinemia. Of the sera examined only those from patients with lupus erythematosus showed significant complement fixation. No significant reaction occurred with sera from 15 normal persons, 21 patients with rheumatoid arthritis, (3 of whom had very high titers of sensitized sheep cell agglutinins), 2 with Laennec's cirrhosis, 3 young patients with cirrhosis and marked hyperglobulinemia, 3 with macroglobulinemia, and 2 with multiple myeloma. In addition, single sera from patients with the following diseases were negative: hemochromatosis, Wilson's disease, infectious mononucleosis, unclassified collagen disease, subacute bacterial endocarditis, acquired hemolytic anemia

TABLE II. Complement Fixation by Representative L.E. Sera with Calf Thymus Nuclei* and Calf Thymus DNA.†

Serum	L.E. cell formation	Antigen	Serum dilution 1	2	4	8	16	32	64	128	256	512	1024
Fr.	Strong	Nuclei	4	4	4	4	4	4	4	4	2	0	0
		DNA	0	0	0	0	0	0	0	0	0	0	0
Ne.	"	Nuclei	4	4	4	4	4	4	1	±	0	0	0
		DNA	4	4	4	4	4	3	0	0	0	0	0
St.	"	Nuclei	4	4	4	4	4	3	0	0	0	0	0
		DNA	4	4	4	4	4	2	0	0	0	0	0
Ga.	Weak	Nuclei	4	4	4	4	3	0	0	0	0	0	0
		DNA	4	4	4	4	4	3	0	0	0	0	0
Hi.	"	Nuclei	4	4	4	4	0	0	0	0	0	0	0
		DNA	4	4	4	4	4	3	0	0	0	0	0

* 2 mg/ml suspension. † 100 γ/ml solution.

with cold agglutinins.

Experiments with DNA: Complement fixation also occurred with L.E. sera and calf thymus, salmon sperm, human leukocyte, and pneumococcal DNA. The degree of fixation with these substances was approximately the same. Most L.E. sera which showed fixation with nuclei also showed a reaction with DNA (Table II). However, serum Fr. was of particular interest because it was a strong L.E. cell inducer and fixed complement to high titer with nuclei, but showed no fixation with DNA. Two other sera with this behavior were encountered, but none was found which was capable of fixing complement with DNA but not with nuclei. Complement fixation with DNA was not observed in a control group of sera similar to those described above for the nuclei studies.‡

Absorption experiments: Cross-absorption with nuclei and DNA was carried out to determine whether the serum factors were identical. Absorption of L.E. serum with nuclei completely removed the ability of serum to induce L.E. cell formation and abolished or reduced complement fixation with nuclei. However, complement fixation with DNA was unaffected. On the other hand, after absorption of serum with DNA, complement fixation with DNA was significantly reduced but L.E. cell formation and fixation of complement with nuclei remained unimpaired (Table III).

TABLE III. Comparison of Effect of Absorption with Nuclei and DNA on Complement Fixation.

Serum	Absorbed with	"Antigen"	Serum dilution 1	2	4	8	16	32	64	128	256	512
Fr.	0	Nuclei*	4	4	4	4	4	4	4	4	1	0
	Nuclei	"	0	0	0	0	0	0	0	0	0	0
	DNA	"	4	4	4	4	4	4	4	4	4	1
	0	DNA†	0	0	0	0	0	0	0	0	0	0
	Nuclei	"	0	0	0	0	0	0	0	0	0	0
	DNA	"	0	0	0	0	0	0	0	0	0	0
Ne.	0	Nuclei*	4	4	4	4	4	4	4	±	0	0
	Nuclei	"	4	4	4	3	1	0	0	0	0	0
	DNA	"	4	4	4	4	4	4	1	0	0	0
	0	DNA†	4	4	4	4	4	4	2	±	0	0
	Nuclei	"	4	4	4	4	4	4	1	0	0	0
	DNA	"	4	2	0	0	0	0	0	0	0	0

* Calf thymus nuclei, 2 mg/ml. † Calf thymus DNA, 100 γ/ml.

‡ L. E. sera which fixed complement with nuclei also reacted strongly with calf thymus nuclear nucleoprotein. Of these sera, two also have fixed complement with histone in low titer.

Experiments with precipitin reaction: Considerable effort was directed toward demonstrating a specific precipitin reaction with DNA. Some of the lupus sera showed ring formation and a precipitate on adding DNA. However, the sera usually had considerable amounts of euglobulin that precipitated readily; the presence of DNA certainly facilitated the precipitation. Two control sera were encountered that also gave a precipitate with DNA. Both of these sera (1 from a patient with cirrhosis, the other from a patient with undiagnosed hyperglobulinemia) failed to give a positive complement fixation test with nuclei or DNA and did not produce L.E. cells. Among the lupus sera, however, a parallelism was noted between the precipitin reaction and complement fixation with DNA.

Discussion. A considerably larger group of patients with miscellaneous disorders will have to be studied before the specificity of the complement fixation with nuclei and with DNA can be clearly established. However, no positive reaction with either material was encountered in conditions other than lupus erythematosus in the present study. It will be of considerable interest to investigate serum from patients with hydralazine sensitivity and certain other disorders known to be associated with positive L.E. tests.

The evidence thus far obtained suggests a correlation between the L.E. test and complement fixation with nuclei. However, absolute comparisons were difficult; the complement fixation reaction is semi-quantitative and may react at high dilutions of L.E. serum while the L.E. phenomenon is difficult to quantitate and even the most active sera cannot be diluted very much without losing activity. In addition, many of the sera used had been stored for varying lengths of time and the ability of some of these sera to form L.E. cells could not be satisfactorily evaluated. Conclusions on this question will require simultaneous complement fixation and L.E. tests on fresh blood, using different methods for assaying L.E. cell formation.

Nuclei from a wide range of organs and species (rabbit leukocytes, calf thymus cells, beef liver cells and human monocytes) gave the complement fixation reaction. Although nuclear constituents probably were responsible for the reactions with these nuclei, a possible role of small cytoplasmic contaminants was not ruled out. The evidence suggested that human leukocyte and calf thymus nuclei were of comparable reactivity, and that this reaction compared favorably in sensitivity with the L.E. test.

The independent behavior of the complement fixation with DNA was unexpected. It had been the working hypothesis that cell nuclei absorbed the L.E. factor and fixed complement because of their content of nucleoprotein or DNA. The present data do not contradict this interpretation; however, the appearance of a separate complement fixing factor for DNA complicates the hypothesis. It is possible that both complement fixing factors are elicited by stimuli from nuclear material, the DNA factor being provoked by more dissociated fragments, and that the resultant factors have different specificities. However, the interrelationship between the serum factors remains unclear. In view of the results with the serum of patient Fr. and also the absorption experiments with other L.E. sera, it is unlikely that the separate DNA complement fixing factor plays a significant role in L.E. cell formation.

Reports of the production of DNA antibodies in experimental animals have appeared in the past(4,5). Other workers(6,7) have described complement fixation reactions with whole cell extracts in lupus erythematosus. These extracts probably contained nuclear materials which may in part have been responsible for the positive results, although cytoplasmic antigens undoubtedly played a role. The present observations suggest that antibodies to certain components of nuclei, including DNA, may be present in the serum of patients with lupus erythematosus. However, the exact nature of the reaction between the serum and nuclear material remains uncertain, and despite the fixation of complement, additional proof that this represents an antigen-antibody reaction is desirable.

Summary. 1. Sera from patients with active lupus erythematosus fixed complement with a wide variety of nuclei from different organs and species, with calf thymus nucleo-

protein, and in two instances with histone. Isolated calf thymus, salmon sperm, human leukocyte and pneumococcal DNA also fixed complement with many of these sera. Similar reactions were not encountered in a limited control series including normal individuals and other pathological states. 2. Most active L.E. sera fixed complement with both nuclei and DNA in roughly parallel titer. However, exceptions were encountered and one serum reacted strongly with nuclei but failed to react with DNA. Cross-absorption experiments with nuclei and DNA suggested the presence of 2 distinct serum factors. 3. The L.E. factor appeared to be related to the factor responsible for complement fixation with nuclei but distinct from that responsible for DNA fixation. 4. The significance of these findings with respect to antibodies against nuclear constituents is discussed.

Addendum: Recent reports by Miescher (*Vox Sanguinis,* 1957, v2, 283) and Seligmann (*Comptes Rendus,* 1957, v245, 1472) also have presented evidence for the presence of antibodies to DNA in lupus erythematosus serum.

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Beneficial Effect of Quinoxaline 1,4-di-N-Oxide in Radiation Injury in Mice.* (23546)

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One of the most prominent features of radiation syndrome in mice is a pronounced bacteremia which appears during the first post-irradiation week and materially contributes to the overall mortality. The type of organisms involved seems to vary with the particular colony of mice used but *Pseudomonas pyocyaneus, Proteus vulgaris, Salmonellae* and various coliform organisms are usually involved. Coulthard and Hale(1) recently showed that quinoxaline-1,4-di-N-oxide was effective in counteracting infection by these organisms in mice. Upon this basis the present investigation was undertaken and the results obtained indicate that, although the drug is not 100% effective, it does materially decrease irradiation bacteremia and total mortality in mice.

Methods. Four hundred male, CF 1 mice, weighing an average of 25 g, were arranged in groups of 20 animals each according to the design given in Table I. The quinoxaline-1,4-di-N-oxide was administered intramuscularly or orally as an acacia suspension in normal saline. Control mice received similar doses of acacia in saline. Four additional groups of 32 animals each were used to determine the effects of the drug on the course of radiation bacteremia and mortality. Eight animals each from medicated and non-medicated groups were sacrificed on post-irradiation days 5, 8, 11 and 14 and the bacterial content of the liver was determined using direct dilutions on Tryptose (Difco) agar. Pure colonies were isolated and identified. The entire small intestine was similarly treated using

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TABLE I. Effect of Oral Quinoxaline-1,4-di-N-Oxide on Radiation Mortality in Mice.

Medication	ST_{50} and range, days	Slope and range	Final mortality %	Final mortality Days
Saline control; day pre-R 3,2,1 *Radiated*	6.4 (5.81– 7.08)	1.25 (1.17–1.34)	100	9
Q 125 mg/kg/day; day pre-R 3,2,1	8.4 (7.5 – 9.41)	1.27 (1.18–1.37)	100	17
Q 250 " ; *idem*			50	68
Saline control; day post-R 1,2,3	7.5 (6.72– 8.37)	1.29 (1.19–1.40)	100	18
Q 125 mg/kg/day; day post-R 1,2,3	9.0 (7.81–10.38)	1.39 (1.26–1.54)	100	16
Q 250 " ; *idem*	7.2 (5.98– 8.68)	1.54 (1.35–1.76)	100	17
Saline control; day post-R 6,7,8	8.5 (7.66– 9.44)	1.27 (1.18–1.37)	100	15
Q 125 mg/kg/day; day post-R 6,7,8	7.9 (7.34– 8.51)	1.19 (1.13–1.26)	100	12
Q 250 " ; *idem*	9 (8.11– 9.99)	1.27 (1.18–1.37)	100	13
Q control; 250 mg/kg/day for 3 days *Non-radiated*	—	—	—	—

ST_{50} = Day on which 50% of animals alive. All values at P, 0.05; 20 animals/group.
Q = Quinoxaline-1,4-di-N-Oxide. Pre-R = Pre-irradiation. Post-R = Post-irradiation.

Liver-Veal agar and Nutrient agar. Total leukocyte and differential counts were performed on 190 additional mice following the drug and/or irradiation. Except during irradiation the animals were maintained in an air-conditioned room at 72 ± 5°F and were fed a diet of Rockland pellets supplemented weekly with additional vit. A and D. The 550 r radiation dose was administered from above and below the mice, with two 250 KVP Picker Industrial Units operating simultaneously. The technical factors were: 250 KVP; 15 ma; FOD 100 cm; filters 0.21 mm Cu inherent, 0.5 mm Cu parabolic and 1 mm AL; HVL 2.02; size of field—total body; r per min. measured in air 17.3 to 18.05. Both units were calibrated before and after each experiment with a Victoreen thimble r-meter. Animals were restrained in a plastic cage similar to the one described for guinea pigs(2). Results of mortality studies were analyzed statistically by the Litchfield method(3).

Results. Table I gives the results of the oral experiment. These data indicate a significant increase in the ST_{50} day in those groups premedicated with either 125 or 250 mg/kg of the drug. With the 250 mg/kg dose there was a marked reduction in total mortality. Post-irradiation medication does not appear to be effective. Similar results were obtained in the first intramuscular experiment in which the total survival for both doses was 35 and 30%/30 days, respectively. Beneficial results were obtained with pre-medication, 250 mg/kg in the second intramuscular experiment using 2 groups of 32 animals each. The ST_{50} day and its range was, controls 10.3 (9.5-11.2) and medicated 20 (16.4-24.4). Total mortality was, controls 100%/14 days and medicated 58%/118 days. The beneficial effects of quinoxaline-1,4-di-N-oxide were reflected in the overall appearance of mice because they showed none of the external signs of radiation injury—rough coat, pale skin and tail, diarrhea, watery eyes and lethargy—exhibited by the controls.

Table II gives the results obtained after culturing liver homogenates of irradiated control and medicated mice. The bacterial counts of livers admittedly measured a greater concentration of bacteria than would have been obtained from heart's blood, and in the later stages of the syndrome probably included organisms from infectional foci. However, all of these sources may be considered

TABLE II. Effect of Quinoxaline-1,4-di-N-Oxide upon Bacteremia in Irradiated Mice.

Post-irrad. sampling day	Bacterial count × 10^3 Control	Bacterial count × 10^3 Medicated
5	25 (2 bfL)	.07 (3 bfL)
8	29	1.5 (1 bfL)
11	441	1.2
14	1584*	.12† (2 bfL)

* Mean of 6 surviving mice.
† Without elimination of 2 of 8 mice having high bacteria counts, the mean would be 100 × 10^3.
bfL—No. of bacteria-free livers.

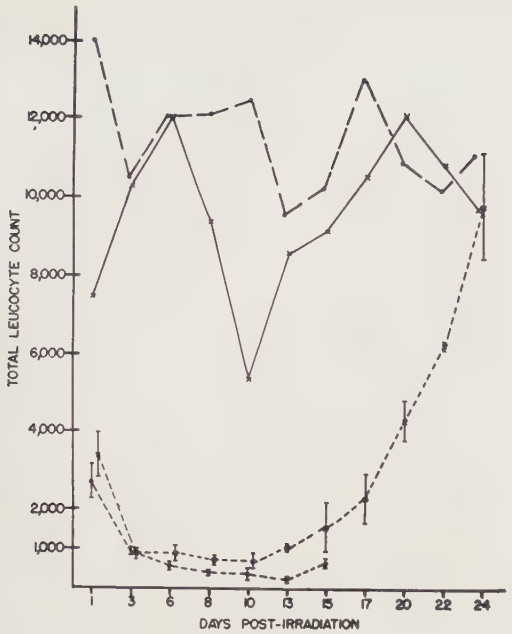

FIG. 1. Effect of irradiation and/or medication on total leukocyte count. Control non-irradiated ----; quinoxaline non-irradiated ×——×; control irradiated —·—·—; quinoxaline irradiated ×----×. Stand. deviations were from 400 to 2100 cells for non-medicated controls, and from 200 to 2000 cells for medicated controls.

to reflect state of infection in the irradiated mouse. The principal invaders in the mouse bacteremia were found to be lactobacilli, coliforms, pseudomonads and streptococci. A saccharomyces-like yeast was discovered as a surprising component of several bacteremic samples.

Because post-irradiation bacteremia has been demonstrated to be largely of enteric origin(4-6), the effect of the drug on small intestine flora was determined using individual plate counts of homogenized entire small intestine from normal animals. The mean bacterial counts from 2 groups of 8 mice each were: control 260.5×10^6 and medicated 15.5×10^6. The order of prevalence of intestinal organisms was as follows: lactobacilli, yeasts of dietary origin, coliforms, streptococci and pseudomonads. Their predominance in mouse bacteremia furnished further evidence of relationship of intestinal flora to irradiation bacteremia and their repression in the small intestine may have furnished part of the chemotherapeutic mechanism accounting for increased survival of irradiated medicated mice. The antibacterial effect of quinoxaline-1-4-di-N-oxide does not appear to be the entire mechanism involved in increasing survival of irradiated mice following 3 days pre-irradiation medication. Comparison of counts of high and low dilutions of liver and intestine homogenates indicated that there were no residues of the drug in the agar plates to influence the growth of bacterial colonies. Thus it appears unlikely that enough of the drug remained in the liver during sampling days to account for the increased survival. However, suppression of enteric organisms would materially decrease the extent of post-irradiation bacteremia thus increasing chances of survival.

The irradiation suppression of the bone marrow of both medicated and non-medicated mice (Fig. 1), indicates that the drug did not influence this phase of the radiation syndrome. However, even during the period of greatest bone marrow depression, the medicated animals always had twice as many circulating leukocytes as the controls. The return to normal leukocyte values in the medicated group follows the pattern described by Lawrence, Dowdy and Valentine(4). The differential counts in all cases reflected the usual picture of radiation injury followed by repair. From the control data in Fig. 1 it appears that quinoxaline-1,4-di-N-oxide caused a preliminary stimulation of the bone marrow followed by a depression then a second stimulation. However, daily leukocyte counts in 10 medicated animals indicated that such fluctuations were artifacts because there were no significant differences in leukocyte values over the control values during the medication days and 2 days post-medication. Moreover, the variation in total leukocytes in the control and medicated non-irradiated mice were within the limits reported by Gardner(5).

Discussion. Quinoxaline-1,4-di-N-oxide is one of the few drugs other than certain antibiotics or sulfhydryl compounds which has a beneficial effect on radiation injury in mice. The data reported indicate that the main beneficial action of the drug is related to its ability to decrease the number of bacteria in the intestinal tract. In this aspect the drug

differs from streptomycin in that it is effective when given prior to and not after irradiation when infection begins. Perhaps the drug also exerts a non-specific beneficial effect such as Treadwell *et al.*(8) demonstrated for estradiol benzoate.

Intestinal lactobacilli formed a formidable component of mouse bacteremia and appeared to have pathological significance in the radiation syndrome in this species. This mouse infection differs from that seen in the rat by Vincent *et al.*(9) where bacteremia lactobacilli counts were relatively low and did not correlate with severe irradiation effects. It is also true that lactobacilli were insignificant or missing from surveys of mouse irradiation bacteremias made by Miller *et al.*(7) and Gonshery *et al.*(10). Differences in lactobacilli concentrations between the present work and that of others(7,9,10) may have been due, in part, to differences in sampling and cultural procedures. Our methods, including homogenization of the liver and culture of the sample in poured, deep-agar plates, favored growth of microaerophilic lactobacilli. Differences in laboratory diet could also have been involved.

Summary. It has been shown that premedication with Quinoxaline-1,4-di-N-oxide is beneficial to irradiated mice, significantly increasing both the ST_{50} day and total number of survivors. The effect has been related to a significant reduction in number of enteric organisms causing the usual irradiation bacteremia. It has been demonstrated that lactobacilli were the predominating invaders under conditions of our experiments. The drug does not appear to have an effect on bone marrow.

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Cardiovascular Responses of Unanesthetized Rats During Traumatic and Endotoxin Shock.* (23547)

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The available information on the direct measurement of arterial pressures in the unanesthetized rat is scant(1,2), and no data have been found where such studies were made during shock from trauma. Chambers *et al.*(3) determined the apparent systolic pressures of unanesthetized rats before and after physical trauma by an indirect method, but they made no systematic presentation of the data.

Thomas(4) recently pointed out the marked similarities of symptoms and pathological changes reported to occur after endotoxin intoxication and traumatic shock, and Schweinburg *et al.*(5) have suggested endotoxins as a factor in shock. It is the purpose of the present investigation to obtain com-

* Dr. David Tennent prepared the sample of endotoxin used.

parative data by direct measurement of the cardiovascular responses of the unanesthetized rat to traumatic shock and to endotoxin intoxication.

Method. White male Wistar rats weighing from 300 to 500 g were used throughout the investigation. Under ether anesthesia a polyethylene catheter, 0.58 mm ID × 7 cm long, was passed subcutaneously to a ventral incision in the neck from a stab wound caudal to the ear. The left carotid artery, carefully separated from the main vagal trunk, was tied centrally and opened; the catheter tip was passed to lie near its root at the aorta and secured by double ligatures. All incised tissues were infiltrated with procaine. This indwelling catheter was connected to a 15-cm length of similar polyethylene tubing by a 1-cm ferrule fashioned from 21 gauge hypodermic tubing. The entire catheter was connected to a Statham strain gauge (P23Da) or a capacitance pressure sensing head (Lilly) by a short 21 gauge needle stub and a 3-way stopcock. The catheter and pressure sensing head were filled with saline-heparin solution. Following closure of the wound, ether administration was discontinued, the animal was placed in a 20 cm × 25 cm × 12.5 cm metal box, and the pressure sensing head was clamped directly overhead. The negative pressure effect of the liquid column above the heart level was less than 5 mm of mercury and was disregarded. Full recovery from anesthesia was assumed after one hour, at which time the animal moved about the box without ataxia. The indwelling catheter was well tolerated and no discomfort was apparent. The arterial pressures were monitored continuously, and permanent recordings were made at appropriate intervals by a photographic oscilloscope or an ink-writing recorder. The respiratory rates were obtained from the arterial pressure records when these waves were reflected clearly in the tracings or by visual counts with a stopwatch. Following control recordings, the catheter was disconnected at the ferrule, open end was plugged, and protruding tip was taped to skin of the neck. The animal was placed in the drum of the Noble-Collip apparatus(6) and was subjected to a standard tumbling, which prior studies(7) had shown to result in a reproducible mortality rate. After trauma the animal was returned to the box, the catheter was connected to the pressure sensing system, and the arterial pressures, heart rates and respiratory rates were recorded in the post-trauma period. Animals alive at the end of observation period were returned to their quarters. All animals surviving 24 hours after trauma were considered survivors. The endotoxin sample was prepared from a strain of *Salmonella newport* and was determined to have an intravenous LD_{50} of 2.8 mg/kg in the rat by the moving average method(8). A dose of 5 mg/kg in saline was injected into the femoral vein of each rat after the arterial catheter had been placed. The ether was removed and the animal was placed in the metal observation box. The arterial pressures, heart rates and respiratory rates were recorded as described. All animals were heparinized after exposure to trauma or endotoxin injection, to prevent occlusion of the catheter. Gross autopsies were performed on all animals immediately following death.

Results. Responses to trauma. Immediately following trauma no significant changes in the arterial pressures or heart rates were observed; however, the respiratory rates rose transiently (Table I). In the 1½-hour period following trauma, the arterial pressures of each animal in the group which eventually died showed a slight but consistent elevation. Those animals subjected to identical trauma but surviving 24 hours evidenced a progressive decline in arterial pressures. Following this rise in arterial pressures in the group dying from trauma, there was a progressive decline in pressures in 4 of the 5 animals until the pre-terminal period. The heart rates and respiratory rates rose significantly as the terminal period was approached. An abrupt fall in respiratory rate usually signified the beginning of terminal collapse (Fig. 1). The respirations became gasping in type. Before the beginning of respiratory collapse the arterial pressures showed no marked hypotension. As the respiratory rate continued to decrease, the arterial pressures declined abruptly to hypotensive levels. Respiratory arrest, preceded by mild convulsive episodes, was fol-

TABLE I. Cardiovascular Responses of Unanesthetized Rats to Drum Trauma and to Endotoxin Intoxication.
5 rats/group.

Time:	Trauma survivors				Trauma deaths			Endotoxin deaths		
	Control	Post-trauma	Mid-period	Final recording	Control	Post-trauma	Mid-period	Pre-terminal	Control (etherized)	Post-inj.	Mid-period	Pre-terminal
Blood pressure, mm Hg												
Systolic	119 ± 18*	129 ± 21	110 ± 12	103 ± 9	123 ± 18	119 ± 30	115 ± 23	119 ± 14	94 ± 15	89 ± 18	95 ± 16	104 ± 16
Diastolic	83 ± 12	86 ± 14	78 ± 10	64 ± 12	80 ± 10	80 ± 16	86 ± 28	84 ± 27	60 ± 29	52 ± 14	54 ± 15	69 ± 17
Avg arterial pressure, mm Hg	102 ± 14	106 ± 16	93 ± 10	82 ± 13	99 ± 22	98 ± 17	100 ± 26	100 ± 25	77 ± 13	70 ± 14	74 ± 13	86 ± 17
Heart rate, beats/min.	324 ± 39	318 ± 31	392 ± 66	399 ± 48	331 ± 52	326 ± 60	411 ± 78	490 ± 82	344 ± 53	370 ± 53	382 ± 43	463 ± 31
Resp. rate, cycles/min.	70 ± 7	103 ± 17	90 ± 16	96 ± 17	78 ± 15	116 ± 34	100 ± 14	134 ± 24	89 ± 6	98 ± 10	90 ± 7	129 ± 13
Survival time	> 24 hr				271 ± 71 min.				270 ± 69 min.			

* ± stand. dev.

lowed by total circulatory collapse with the heart continuing to beat for a few minutes. The respiratory and heart rates of the surviving group were elevated only slightly by comparison, while the arterial pressures remained slightly below the pre-trauma levels.

FIG. 1. Cardiovascular and respiratory responses of unanesthetized rat to fatal drum shock.

FIG. 2. Cardiovascular and respiratory responses of rat to a lethal intrav. inj. of endotoxin. Light ether anesthesia was discontinued at zero time.

FIG. 3. Central arterial pulse contours of unanesthetized rats recorded in control period and during early pre-terminal period after standard drum trauma and lethal doses of endotoxin. (A) Photographic recorder (retouched). (B) Ink-writing galvanometer.

* Shaded area.

Fig. 3 presents the individual pressure pulse contours of the unanesthetized rat in the control period and in the pre-terminal period after fatal trauma. Relative to the control, the post-trauma contours show shortened cycle durations, curtailed systolic ejection times, collapse of the pressure curve from mid-systole to aortic valve closure, and a slight positive pressure wave in the diastolic limb. Such changes are indicative of decreased venous return, compensatory vasoconstriction and cardio-acceleration. Cardiac contractility, as indicated by the slope of early systolic upstroke, did not appear markedly altered. Arrhythmias, as indicated by the regularity of the pulses, did not appear until respiratory failure occurred. The pulse contours of rats surviving trauma showed similar but less marked changes, and at the end of the observation period the contours resembled those recorded prior to trauma.

Responses to endotoxin. It may be seen in Table I that the control arterial pressures of this group of rats were on the average lower than the group subjected to trauma, possibly the result of ether anesthesia. The immediate response of the arterial pressures of the etherized rats to the injection of endotoxin was insignificant, although one animal sustained a transient fall of 24 mm Hg. For ½ hour following injection, there was a mixed response of recovery from anesthesia and of endotoxin effect. A moderate depressor response was observed to reach a maximum approximately 1½ hours after injection, the diastolic pressures declining more than the systolic pressures (Fig. 2). This delayed vasodepression also was observed in the anesthetized rat by Zweifach *et al.*(9). Following this response there was a sustained and gradual rise in pressures, with a narrowing of pulse pressures until terminal collapse. The average arterial pressures at the pre-terminal period were lower than the corresponding pressures of the group dying from trauma. Yet an average pressure of 86 ± 17 mm Hg does not indicate hypotension as a major factor contributing to the collapse. The sequence of events at terminal collapse was similar to those described for animals dying from trauma. The pulse contours, as recorded in the pre-terminal period, while differing somewhat, show basically the same changes that were recorded in rats subjected to trauma (Fig. 3).

Gross observations. The gross reactions of rats following fatal trauma and endotoxin injection were similar. Both groups of animals exhibited decreased physical activity, somnolence, pilo-erection, bloody stools and pale, cool extremities. Rectal temperatures were usually subnormal; yet, a hyperthermia was evident when recordings were obtained from the upper descending colon. Urination was uncommon, and the animals often appeared hyper-reactive to auditory or pinch stimuli. The respiratory failure and convulsive episodes were suggestive of hypoxic involvement. The gross changes at post mortem of rats dying from trauma were essentially the same as those described by Noble and Collip(6). Similar pathologic changes in the visceral organs were observed in rats dying from lethal doses of endotoxin, except that the endotoxin-treated rats exhibited petechial hemorrhages in the small bowel, the cecum rarely being involved, which contrasts with the large dark confluent hemorrhagic areas in the small bowel and the cecum observed after traumatic deaths. Pathologic changes in the colon and rectum were not observed after either type of death.

Discussion. The systolic and diastolic pressures of unanesthetized rats were lower on the average than those reported previously(1). However, the heart rates also were considerably lower, indicating that the lack of restraint may have contributed to these differences. The damping effect of the catheter on the frequency response of the recording system is acknowledged; yet, the small volume displacement per pressure increment of the pressure sensing manometers would tend to minimize this error. The average arterial pressures, which differed by less than 10% from the electronically recorded mean pressures, should be relatively accurate values.

The absence of a marked hypotension in rats subjected to fatal trauma was surprising, since such a finding is usually associated with the state of shock. Undoubtedly, the powerful vasoconstrictor response of the rat(2) maintains the central arterial pressures in the

face of a rapidly developing circulatory deficit. While a transient marked hypotensive response was reported previously to occur following trauma in the rat(3), inherent factors in the indirect method(10) coupled with reported difficulties of determining pressure in peripheral arteries in a state of constriction (11) may have been operative. The progressive rise in the central arterial pressures of rats during the 1½-hour period after trauma as observed here is probably a reflection of the intense sympatho-adrenal discharge demonstrated by Young and Gray(12) in the rat after trauma.

The cardiovascular responses of the rat to lethal doses of endotoxin, while differing in several aspects, were not markedly different from the responses evoked by fatal trauma. In both groups of rats, evidence of sympathetic activity, tachycardia, tachypnea, circulatory deficit and similar patterns of collapse at terminus were observed. The endotoxin group differed by showing transient vasodepressor responses, lower arterial pressures throughout and the absence of gross pathological changes in the cecal tissues. The large dose of endotoxin and the absence of physical trauma may account for some of these observed differences.

Profound hypotensive episodes have been reported to immediately follow the intravenous injection of endotoxins in the rabbit(13), cat(14) and dog(15). These responses resembled a histamine-release phenomenon in that tachyphylaxis developed(13). The relative absence of this immediate response in the rat may be a species difference.

The immediate cause of the terminal failure as indicated by the initial collapse of respiratory function implied a primary failure of brain stem function. Such views also have been held responsible for the terminal collapse in hemorrhagic shock in this species(2). However, this functional failure following trauma or endotoxin does not appear to be related directly to the level of central arterial pressure, but more likely to curtailment of effective peripheral blood flow and oxygen exchange at the vital centers.

In general, the studies presented here provide evidence that the various cardiovascular responses, symptoms and pathologic changes in rats subjected to endotoxin intoxication are similar in several aspects to those responses observed in this species subjected to lethal trauma. While certain differences have been observed in these responses, they are considered of insufficient magnitude to exclude endotoxins as possible factors concerned with the irreversible processes of traumatic shock. However, as attractive as the proposals are that endotoxins are operative in traumatic shock, Thomas(16) has reemphasized caution in the interpretation of like responses to dissimilar stresses as involving common factors. At the present, direct evidence is lacking.

Summary. The arterial pressures, heart rates and respiratory rates of unanesthetized rats have been recorded by direct measurement. The effects of traumatic shock and lethal dosages of endotoxin on these responses have been observed. The cardiovascular responses, symptoms and pathologic changes observed in rats subjected to trauma and to endotoxin intoxication were similar and, while certain differences have been observed, they are considered to be of insufficient magnitude to exclude endotoxins as possible factors concerned with their proposed role in irreversible shock.

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Intrinsic Factor Studies VI. Competition for Vit. B_{12} Binding Sites Offered by Analogues of the Vitamin.* (23548)

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The mechanism by which intrinsic factor, a component of normal human gastric juice, enhances absorption of vit B_{12} from the gut has not yet been elucidated. Recently reported evidence(1,2) indicates that vit B_{12} which is bound to gastric juice is absorbed preferentially over unbound vitamin. This observation suggests that binding of vit B_{12} is an essential property of intrinsic factor. However, the ability to bind vit B_{12} is an attribute of several biologic substances which do not possess intrinsic factor activity(3-7). As far as we are aware, there are no published reports of preparations possessing intrinsic factor activity but lacking the property of binding B_{12}. It is possible that binding of vit B_{12} by intrinsic factor is a unique process as compared to binding of the vitamin by substances lacking intrinsic factor activity. To determine if the vit B_{12} binding mechanism of intrinsic factor is more fastidious than that of other vit B_{12} binding proteins, we have measured competition offered by certain vit B_{12} analogues for vit B_{12} binding sites of several biologic fluids. This is a report of such observations.

The effect of an excess of one vit B_{12} analogue, pseudovit B_{12}, on radioactive vit B_{12} binding by human gastric juice has been reported(7). Binding of vit B_{12} was only slightly reduced in the presence of a 50-fold excess of the pseudovitamin. It was concluded, then, that this analogue presents little competition for vit B_{12} binding sites of gastric juice. Gregory and Holdsworth(8) have made somewhat analogous observations on preferential binding of vit B_{12} by an intrinsic factor preparation derived from hog mucosa.

Materials and methods. Vit B_{12} analogues. Ten crystalline analogues of vit B_{12}† were studied. Three of the analogues,‡ sulfatocobalamin (SU-B_{12}), nitrocobalamin (NI-B_{12}), and chlorocobalamin (CL-B_{12}), hold sulfate, nitrate, or chloride ions, respectively, in lieu of the cyanide ion(9). In 4 other B_{12} analogues, desdimethylbenziminazole B_{12} (DDMBI-B_{12}),§ 5(6)-trifluoromethylbenziminazole B_{12} (TFMBI-B_{12}),§ 5(6)-hydroxybenziminazole B_{12} (HBI-B_{12})‖ or Factor III, and 5(6)-aminobenziminazole B_{12} (ABI-B_{12}),§ the side chains on the benziminazole moiety differ from those 2 methyl groups filling comparable loci in B_{12}(10,11). If adenine occupies the position filled by 5,6-dimethylbenziminazole in the B_{12} molecule, the analogue is known as pseudovit B_{12}(pseudo-B_{12})¶ (12,13), the sample of which used for this project was entirely free of B_{12}. Lactam (lactam-B_{12})§ and lactone (lactone-B_{12})§ forms result from cyclization of the acetamide

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† Vit. B_{12} (cyanocobalamin) = B_{12}.

‡ Kindly donated by C. Rosenblum, Merck and Co.

§ Kindly donated by E. L. Smith and K. E. Fantes, Glaxo Labs., England.

‖ Kindly donated by K. Bernhauer, Aschaffenburger Zellstoffwerke AG., Germany.

¶ Kindly donated by J. J. Pfiffner, Parke, Davis, and Co.

TABLE I. Failure of $B_{12}Co^{60}$ Molecules to Dissociate from Gastric Juice (GJ) Binding Sites in Presence of Excess Non-radioactive Vitamin.

Aliquot	Order of addition (x = 10 mμg/ml)	% retention $B_{12}Co^{60}$ after dialysis
1	1x $B_{12}Co^{60}$ GJ*	96
2	1x $B_{12}Co^{60}$ 50x B_{12} GJ*	7.9
3	50x B_{12} GJ* 1x $B_{12}Co^{60}$*	4.5
4	1x $B_{12}Co^{60}$ GJ* 50x B_{12}*	87
5	1x $B_{12}Co^{60}$ 50x B_{12} H_2O	.2

* One hr of equilibration at room temperature followed addition of this substance. Refer to Fig. 1 for B_{12} binding capacity.

chain on ring B of B_{12}. The preparation of the 2 latter compounds, free from traces of the other or of B_{12}, has been described(14). Analogues SU-B_{12}, NI-B_{12}, CL-B_{12}, and pseudo-B_{12} were weighed on a micro-balance, dissolved in fresh 0.85% saline, and autoclaved. DDMBI-B_{12}, TFMBI-B_{12}, HBI-B_{12}, lactam-B_{12}, and lactone-B_{12} were dissolved in aqueous 0.5% phenol and the optical density of each solution determined in a Beckman DU Spectrophotometer at 361 mμ. The concentration of each analogue was calculated on the assumption that the $E^{1\%}_{1\,cm}$ at 361 mμ is the same as that for B_{12}. Only one of the analogues, ABI-B_{12}, was received in solution. When adding 50-fold amounts of analogues, their molecular weights were considered to equal that of B_{12}. The same B_{12} analogue stock solutions were used throughout the observations reported herein. *Biologic substances.* Gastric juice was collected after histamine stimulation from at least 15 donors, pooled, and processed as previously described (15). Boiled gastric juice was prepared by placing a sample in a test tube in boiling water for 10 minutes. Saliva was collected from one person prior to meals and filtered through coarse sintered glass. Colostrum was collected from 7 post-partum mothers and pooled. Liver and kidney extracts were prepared by homogenizing the tissue in water in a high speed grinder-mixer. The supernate from such a homogenate was dialyzed against water. Liver extract required centrifugation and filtration after dialysis to yield a relatively clear preparation. Intrinsic factor concentrate (IFC)** was from hog gastric mucosa. All of these materials, when in solution, were stored at –12°C. *Assay.* Binding of radioactive B_{12}†† by biologic substance was determined quantitatively by comparing amounts of radioactivity before and after dialysis in Visking tubing against running tap water for 60 hours(7). The effect of excess analogue on B_{12} binding was tested by adding a sample of biologic material to a tube containing a measured amount of radioactive B_{12} (1 ×) and 50 times that amount of B_{12} analogue (50 ×). This order of addition of materials was varied for the experiments documented in Table I. By observing per cent retention of radioactive B_{12} and by comparing this percentage with that in a "control" tube; *i.e.,* a tube containing biologic material and 1 × radioactive B_{12} only, it was known to what extent excess analogue had affected B_{12} binding. When water rather than biologic substance was added to the same amount of 1 × $B_{12}Co^{60}$ and 50 × B_{12}, retention of $B_{12}Co^{60}$ after dialysis was less than one per cent.

Results. I. *B_{12} binding capacity.* When increasing amounts of B_{12} are added to gastric juice, the amount of B_{12} bound increases until a maximum or plateau is reached (Fig. 1). When large excesses of B_{12} are added, the binding capacity is slightly increased. Boiling gastric juice reduces markedly, but does not eliminate, binding of B_{12} (Fig. 1). The B_{12} binding curves for saliva, colostrum, serum, and aqueous liver and kidney extracts are shown in Fig. 1 and 2. Saliva binds as much B_{12} per mg protein as does gastric juice but it is known(16) that saliva does not act as intrinsic factor. The B_{12} binding curve of liver or kidney is a straight line rather than an

** Supplied by H. Wijmenga, N. V. Organon, Oss, Holland.

†† Radioactive B_{12} = $B_{12}Co^{60}$, a portion of which was donated by C. Rosenblum, Merck & Co.

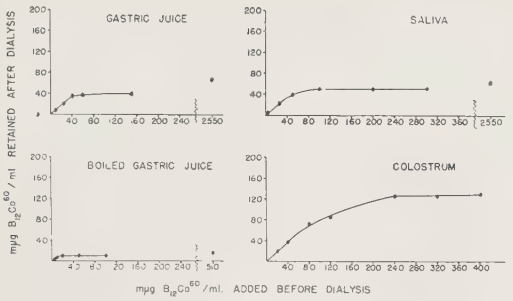

FIG. 1. Vit. B_{12} binding capacity of pooled neutralized normal human gastric juice (Biuret protein = 1.2 mg/ml), saliva (Biuret protein = 0.9 mg/ml), and colostrum (Biuret protein = 77 mg/ml).

ascent to a plateau. In accord with a report by Bird and Hoevet(3) lysozyme does not prevent B_{12} from passing out of the dialysis bag.

II. *Stability of B_{12} binding.* To determine the degree of dissociability of gastric juice-bound B_{12} in the presence of excess vitamin, the experimental plan outlined in Table I was devised. It is apparent that, given a chance for prior binding, most of the $B_{12}Co^{60}$ molecules remain bound even in the presence of a 50-fold excess of non-radioactive vitamin (aliquot 4). If there had not been any exchange of vitamin molecules in this aliquot, however, per cent retention would have equalled that in aliquot 1. Postdialysis radioactivity in aliquot 3 was less than in the second test aliquot. If there had not been *any* exchange of vitamin molecules in the third aliquot, essentially no radioactivity would have survived dialysis. The amount of B_{12} added, 500 mμg/ml, greatly exceeded the binding capacity of gastric juice, and thus should have prevented binding of $B_{12}Co^{60}$ added subsequently. The differences between the data in aliquots 2 and 3 are reproducible. A *second experiment,* which strengthened this observation, was done as follows: An amount of $B_{12}Co^{60}$ known to saturate all B_{12} binding sites at the 1 × level (40mμg/ml) was added to gastric juice samples and allowed to equilibrate at room temperature for one hour. Next, 50 times that amount of non-radioactive B_{12} was added and allowed to equilibrate at 4°C for varying lengths of time, 2 minutes to 71 hours, before dialysis. (The low temperature was maintained to avoid possible deterioration of intrinsic factor.) Retention of $B_{12}Co^{60}$ was not lessened as the time of equilibration before dialysis was increased. Stated in another way, these results demonstrate that complete equilibrium between previously bound $B_{12}Co^{60}$ and a subsequently added excess of non-radioactive B_{12} occurred within a few minutes after addition of the latter.

III. *Competition for B_{12} binding sites by analogues.* If the amount 1 × $B_{12}Co^{60}$ is not sufficient to saturate all B_{12} binding sites of gastric juice, competition for those sites offered by an analogue may not be manifest. This was clearly seen in one experiment, the results of which are listed in Table II. It has been established that pseudo-B_{12} offers slight competition for B_{12} binding sites of gastric juice(7). However, when the amount of $B_{12}Co^{60}$ added was not sufficient to saturate the B_{12} binding sites, competition by pseudo-B_{12} was not observed. (Compare aliquots 1 and

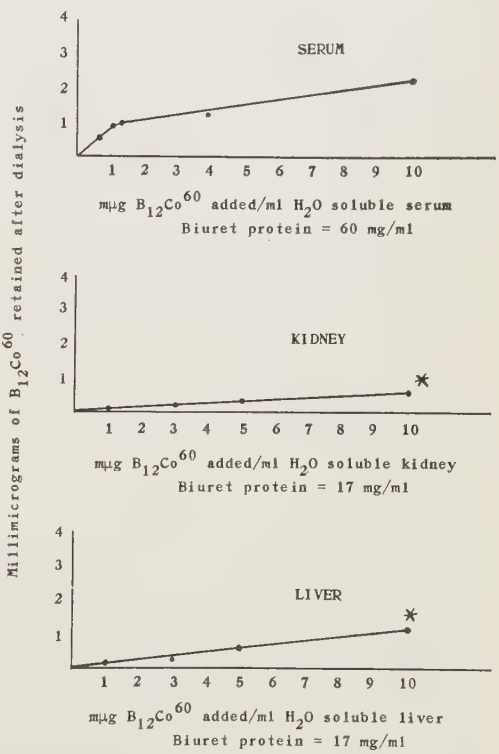

FIG. 2. B_{12} binding by normal human serum and aqueous kidney and liver extracts.

* Slope is similar when abscissa is extended to 100 mμg/ml.

TABLE II. Variation in Competition Offered by Pseudo-B_{12} for B_{12} Binding Sites of Gastric Juice (GJ) Depending upon Amount of $B_{12}Co^{60}$ Added.

Aliquot	Substances to which GJ* added: 1x $B_{12}Co^{60}$ (mμg/ml)	50x pseudo-B_{12} (mμg/ml)	% retention $B_{12}Co^{60}$ after dialysis	Calculated competition, %†
1	10		85	
2	10	500	85	.9
3	25		85	
4	25	1250	83	2.4
5	40		87	
6	40	2000	67	22
7	60		61	
8	60	3000	49	20
9	150		26	
10	150	7500	21	21

* Refer to Fig. 1 for B_{12} binding capacity.
† See text for calculation.

2; 3 and 4.) When the B_{12} binding sites were saturated, reduced retention of $B_{12}Co^{60}$ in the presence of excess pseudo-B_{12} resulted. (Compare aliquots 5 and 6.)

Included in Tables II, IV, and V, are figures for "calculated competition." This is a numerical expression of per cent reduction of the quantity of $B_{12}Co^{60}$ bound in the presence of excess analogue. The calculation is as follows:

Let A = mμg $B_{12}Co^{60}$ bound in *absence* of analogue

Let B = mμg $B_{12}Co^{60}$ bound in *presence* of analogue

Then $\frac{A-B}{A} \times 100 =$ calculated competition %

From such calculations a constant value for competition is obtained in spite of increasing quantities of $B_{12}Co^{60}$. (Compare aliquots 6, 8 and 10 of Table II.) Similar observations were noted in other experiments. Thus, it was necessary to determine B_{12} binding capacity of each biologic sample prior to testing competition offered by B_{12} analogues for the B_{12} binding sites. The binding capacity is considered as that point at which there is a sharp break in the binding curve determined as in Fig. 1.

A. *Gastric juice.* Competition for B_{12} binding sites of gastric juice offered by SU-B_{12}, NI-B_{12} and CL-B_{12} was investigated. Representative data, comprising Table III, demonstrate that competition by these analogues for the B_{12} binding sites is similar to that offered by excess B_{12}. Further evidence for the similarity of binding of SU-B_{12} and B_{12} by gastric juice was obtained from the observation that binding of radioactive sulfatocobalamin‡‡ resembled binding of $B_{12}Co^{60}$; both the slope of the curve and the maximum amount bound were similar. Results of studying competition for B_{12} binding sites of gastric juice offered by DDMBI-B_{12}, TFMBI-B_{12}, HBI-B_{12}, ABI-B_{12}, lactam-B_{12}, lactone-B_{12} and pseudo-B_{12} are listed in Table IV. These analogues present differing amounts of competition for the B_{12} binding sites, pseudo-B_{12} offering least competition. Lactam-B_{12} and lactone-B_{12} are poor competitors. Excess non-radioactive B_{12} does not offer 100% calculated competition for the B_{12} binding sites because observed binding capacity increases slightly with higher vitamin concentration (Fig. 1).

B. *Intrinsic factor concentrate.* The analogues are more effective competitors for the binding sites of the hog mucosal preparation than for those of human gastric juice (Table V).

C. *Saliva.* Most of the analogues present competition similar to that offered by B_{12} for the B_{12} binding sites (Table IV).

D. *Colostrum.* Pseudo-B_{12} is not as successful a competitor for the B_{12} binding sites as is DDMBI-B_{12} or B_{12} but, yet, is a more effective one than in gastric juice (Table IV).

TABLE III. Competition for B_{12} Binding Sites of Gastric Juice (GJ) Offered by SU-B_{12}, NI-B_{12}, or CL-B_{12}.

Aliquot	Substances to which GJ added: 1x* $B_{12}Co^{60}$	50x B_{12}	50x SU-B_{12}	50x NI-B_{12}	50x CL-B_{12}	% retention $B_{12}Co^{60}$ after dialysis
1	+	—	—	—	—	82
2†	+	+	—	—	—	2.6
3	+	—	+	—	—	3.2
4	+	—	—	+	—	2.5
5	+	—	—	—	+	2.7

* x = 50 mμg/ml GJ. Refer to Fig. 1 for B_{12} binding capacity.
† When water rather than GJ was added to the same amounts of 1x $B_{12}Co^{60}$ and 50x B_{12}, % retention of $B_{12}Co^{60}$ after dialysis equalled 0.3.

‡‡ Kindly donated by C. Rosenblum.

TABLE IV. Analogue Competition for B_{12} Binding Sites of Certain Biologic Substances.

Aliquot	Substances to which biologic sample added 1x	50x	Gastric juice* (x = 40 mμg/ml) % retention $B_{12}Co^{60}$ after dialysis	Calc. competition, %	Saliva* (x = 50 mμg/ml) % retention $B_{12}Co^{60}$ after dialysis	Calc. competition, %	Colostrum* (x = 120 mμg/ml) % retention $B_{12}Co^{60}$ after dialysis	Calc. competition, %	Serum† (x = 1 mμg/ml) % retention $B_{12}Co^{60}$ after dialysis	Calc. competition, %
1	$B_{12}Co^{60}$		88		91		86		94	
2	"	B_{12}	2.6	97	2.4	97	3.6	96	9.5	90
3	"	DDMBI-B_{12}	7.9	91	2.7	97	3.9	95	11	88
4	"	TFMBI-B_{12}	11	87	2.6	97			9.2	90
5	"	HBI-B_{12}	13	85	3.7	96			11	88
6	"	ABI-B_{12}	16	82	3.6	96			15	84
7	"	lactam-B_{12}	30	66	12	87	15	83	33	65
8	"	lactone-B_{12}	61	30	10	90	26	69	53	44
9	"	pseudo-B_{12}	72	18	5.6	94	17	81	9.2	90

* Refer to Fig. 1 for B_{12} binding capacity.

† Refer to Fig. 2 for B_{12} binding.

E. *Serum.* Pseudo-B_{12} offers as much competition as does B_{12} for the binding sites of serum (Table IV).

Discussion. If binding of B_{12} in the presence of excess analogue is not changed, then that analogue is not offering any competition for the B_{12} binding sites. The analogue, pseudo-B_{12}, added in 50-fold excess of $B_{12}Co^{60}$, offers roughly 20% competition for B_{12} binding sites of gastric juice in most cases. It is known that the only difference between pseudo-B_{12} and B_{12} is that the 5,6-dimethylbenziminazole moiety of the latter is replaced by adenine(12,13). This modification strikingly reduces the ability of the molecule to compete for B_{12} binding sites of gastric juice. When the benziminazole moiety is present in the molecule but lacks the 2 methyl groups at positions 5 and 6 (DDMBI-B_{12}), there is almost complete competition for the B_{12} binding sites (Table IV). Rosenblum, Davis, and Chow(18) have recently reported that DDMBI-B_{12} is very poorly absorbed in man. Therefore, it is apparent that the great selectivity of absorption of cyanocobalamin by the human is not solely dependent upon selective binding by intrinsic factor.

TABLE V. Analogue Competition for B_{12} Binding Sites of Intrinsic Factor Concentrate (IFC).

Aliquot	Substances to which IFC added 1x*	50x	% retention $B_{12}Co^{60}$ after dialysis	Calc. competition, %
1	$B_{12}Co^{60}$		60	
2	"	B_{12}	1.4	98
3	"	DDMBI-B_{12}	2.0	97
4	"	TFMBI-B_{12}	2.3	96
5	"	HBI-B_{12}	2.0	97
6	"	ABI-B_{12}	2.0	97
7	"	lactam-B_{12}	5.0	92
8	"	lactone-B_{12}	6.2	89
9	"	pseudo-B_{12}	6.2	89

* x = 30 mμg/0.01 mg IFC.

The fact that boiled gastric juice, known to lack intrinsic factor activity, maintains considerable preference for B_{12}(7) is evidence that selective binding of B_{12} is not equivalent to intrinsic factor activity. One possible explanation is that the intrinsic factor molecule has several functional groups which are necessary for physiologic activity. The binding may be due to a portion of the molecule which is only partially affected by boiling. The decreased binding capacity of boiled gastric juice may result from covering of some of the sites during boiling.

The B_{12} binding sites of saliva, colostrum, and serum exhibited markedly less preference for B_{12} in the presence of a 50-fold excess of pseudo-B_{12} than did those of gastric juice (Table IV). In fact, pseudo-B_{12} and B_{12} are equally successful competitors for B_{12} binding sites of saliva and serum. These data are interpreted as demonstrating qualitative differences in the B_{12} binding substances of these sources. It is known that these 3 substances lack intrinsic factor activity(2,16,17).

Another possibility is that each biologic material known to bind B_{12} contains a different ratio of "specific" and "non-specific" B_{12} binding substances, in some cases the latter exceeding the former to such a degree that

any specificity for B_{12} is masked. This conjecture might explain why differing preference patterns for B_{12} in the presence of certain B_{12} analogues were expressed by gastric juice and IFC (Tables IV and V). Discrepancies between B_{12} binding and intrinsic factor activity may be due to varying amounts of B_{12} binding substances other than intrinsic factor.

Summary and conclusions. 1) This article reports some observations on the general nature of vit B_{12} binding by gastric juice and certain other biologic substances. The majority of vit B_{12} molecules bound by gastric juice did not dissociate in the presence of excess vitamin molecules added subsequently. 2) Our data show competition offered by certain vit B_{12} analogues for the cyanocobalamin binding sites of gastric juice, intrinsic factor concentrate, saliva, colostrum, and serum. Of these substances, gastric juice exhibited the greatest preference for cyanocobalamin in the presence of excess analogue. The intrinsic factor concentrate tested was much less fastidious than gastric juice. Saliva, colostrum, and serum exhibited little selectivity in their B_{12} binding mechanisms. 3) Occurrence of a sulfate, nitrate, or chloride ion in lieu of the cyanide ion in the cobalamin did not diminish competition for cyanocobalamin binding sites of gastric juice.

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Effects of Sodium and Potassium upon Cardiac Glycogen Fractions of the Rat Heart.*† (23549)

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A medium low in sodium and potassium stimulates glycogenesis in the rat heart(1) and similarly, a high potassium concentration initiates glycogenolysis(2). It has also been reported that a solution low in potassium will

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TABLE I. Effects of Sodium Ion upon Fractional Glycogen Synthesis in Rat Heart. Glucose osmolarity in solution = .055 except where indicated.*

Series	No. of determinations	Na (osmols)	Total osmolarity of solution	Glycogen as glucose (μM/g) Acid-insoluble	Acid-soluble	Total	O_2 consumption (μM/g wet wt/hr)
Controls	100			6.11 ± .155†	3.09 ± .137†	9.50 ± .257†	
Exp. 1-14	16	.00	.300*	3.95 ± .296	16.29 ±1.177	20.30 ±1.228	44.5
	19	.01	" *	4.38 ± .453	10.06 ± .589	14.75 ± .907	46.5
	16	.00	"	3.33 ± .289	15.05 ± .848	18.32 ± .999	30.9
	26	.02	"	3.15 ± .179	4.94 ± .210	8.02 ± .358	33.2
	31	.05	"	3.58 ± .310	8.39 ± .406	11.97 ± .605	38.0
	21	.06	"	2.90 ± .176	5.86 ± .460	8.70 ± .611	33.6
	22	.10	"	3.27 ± .236	6.11 ± .833	9.32 ±1.012	37.0
	17	.13	"	2.65 ± .216	1.97 ± .211	4.63 ± .289	27.3
	8	.20	"	2.65 ± .275	2.10 ± .205	4.81 ± .477	21.9
	15	.29	.345	2.16 ± .335	2.34 ± .360	4.63 ± .722	19.7
	15	2.00	2.055	2.65 ± .165	2.28 ± .146	4.81 ± .231	0.0
	9	.02	.345	1.73 ± .193	2.65 ± .370	4.38 ± .463	25.3
	5	"	.400	2.47 ± .548	3.09 ± .436	5.55 ± .950	26.9
	6	"	.450	2.34 ± .565	3.33 ± .532	5.24 ± .949	23.1

* Glucose osmolarity = .287. † ± S.E. of mean.

produce glycogenolysis(3,4). Poppen, Green, and Wrenn(5) point out that if the glycogen content of the heart is high the potassium ion concentration will likewise be high. Experiments of a similar nature have been performed with other tissues in an effort to explain total glycogen changes. The results reported are variable presumably because tissues have permeability differences and respond differently in the same ionic environment. Marsh and Miller(6) demonstrated glycogen synthesis with rat kidney slices in a potassium medium. This is in general agreement with the results obtained by Hastings(7), Buchanan, Hastings, and Nesbett(8) and Hastings, Teng, Nesbett, and Sinex(9) who found that a potassium medium was best for glycogen synthesis from glucose by rat liver slices. Sodium had an inhibitory effect upon glycogen synthesis or stimulated the glycogenolytic processes. Stadie and Zapp(10) observed glycogenesis with rat diaphragm tissue in a sodium medium.

The preceding experiments involve total glycogen only and indicate it is influenced considerably by changes in the electrolytic pattern. It has been established that glycogen exists in more than one form and in this paper the effects of variable sodium and potassium concentrations upon these glycogen fractions of the rat heart were investigated.

Materials and methods. Male and female adult white rats, unfasted, were decapitated and the heart quickly excised. The great vessels and auricular tissue were discarded. The ventricular tissue was placed in a Stadie-Riggs tissue slicer and slices approximately 0.5 mm in thickness and weighing between 45 and 65 mg were prepared. Each slice was placed in a Warburg flask containing the appropriately gassed solution. Two or more slices were weighed quickly and then frozen between blocks of dry ice for later analysis of reference control levels of the myocardial glycogen fractions. Standard Warburg procedures were employed and the tissue in the flasks was shaken for 2 hours. Oxygen utilization was recorded every one-half hour. When the flasks were removed, tissue from 2 flasks was combined in order to insure adequate mass for glycogen analysis. The tissue was homogenized immediately in cold 10% trichloracetic acid and the acid-soluble glycogen determined by a procedure described by Bloom, Lewis, Schumpert, and Shen(11). The acid-insoluble glycogen fraction was determined by digesting the residue, obtained by centrifugation of the soluble moiety, in hot 30% KOH. Both glycogen fractions subsequently were hydrolyzed in 95% sulfuric acid and quantitatively determined by the anthrone method as reported by Seifter, Dayton, Novic, and Muntwyler(12). The *media* used may be noted in Tables I and II. In most experimental situations the osmolarity of the solution was maintained at 0.300. With the

TABLE II. Effects of Potassium Ion upon Fractional Glycogen Synthesis in Rat Heart. Glucose osmolarity in solution = .055 except where indicated.* Sodium osmolarity in solution = .020 except where indicated.†‡

Series	No. of determinations	K (osmols)	Total osmolarity of solution	Glycogen as glucose (μM/g) — Acid-insoluble	Acid-soluble	Total	O_2 consumption (μM/g wet wt/hr)
Controls	100			6.11 ± .155§	3.09 ± .137§	9.50 ± .257§	
Exp. 1-10	16	.000†	.300*	3.95 ± .296	16.29 ±1.177	20.30 ±1.228	44.5
	16	.000†	"	3.33 ± .289	15.05 ± .848	18.32 ± .999	30.9
	19	.000‡	" *	4.38 ± .453	10.06 ± .589	14.75 ± .907	46.5
	12	.012	"	3.15 ± .243	6.60 ± .352	9.69 ± .356	37.2
	7	.025†	"	2.41 ± .179	5.55 ± .286	7.96 ± .398	32.7
	12	.025	"	2.96 ± .215	4.81 ± .495	7.77 ± .666	39.2
	11	.050	"	2.22 ± .111	3.52 ± .178	5.61 ± .195	38.2
	8	.220	"	1.48 ± .182	2.10 ± .158	3.58 ± .306	25.5
	4	.400	.475	1.11 ± .154	1.85 ± .229	2.96 ± .358	.0
	2	2.460	2.535	1.05 ± .215	1.67 ± .062	2.71 ± .151	.0

* Glucose osmolarity = .287. † Sodium osmolarity = .000. ‡ Sodium osmolarity = .010.
§ ± S.E. of mean.

appropriate electrolyte and glucose osmolarities, the total osmolarity desired was obtained by the addition of sorbitol, a non-ionic, metabolically inert compound(1).

Results. Sodium. The information presented in Table I indicates absolute values and allows one to compare raw data. Statistical analysis of the data shows there is a decrease in the acid-insoluble glycogen fraction in all experimental conditions when compared to the control animals. The P value in all cases was $<.001$. The effects observed with the acid-soluble fraction are more diversified. There is a significant increase ($P = <.001$) in this glycogen fraction when the experimental medium contained no sodium or sodium in the amounts of 0.010 to 0.100 osmol (Table I, Exp. 1 through 7). A solution with a sodium concentration of 0.130 osmol or more (Exp. 8 through 11) or a condition in which the osmolarity of the solution is increased (Exp. 12 through 14) does not significantly alter the level of the acid-soluble fraction. Total glycogen, obtained by the algebraic sum of the 2 fractions, is significantly different ($P = <.001$) from the control levels in all experiments except 6 and 7.

In these particular experiments oxygen utilization was higher in solutions containing 0.287 osmol of glucose or containing 0.100 osmol or less of sodium than in a more concentrated sodium medium.

Potassium. When the acid-insoluble component of the experimental series was compared statistically to the reference control values there was a significant decrease in this glycogen fraction ($P = <.001$) in all cases (Table II). The changes observed in the acid-soluble fraction followed a rather precise pattern. Solutions containing from zero to 0.025 osmol of potassium (Exp. 1 through 6) produced a significant increase in this glycogen fraction when compared to the control value ($P = <.001$). There was no significant difference when the solution contained 0.050 osmol or more of potassium (Exp. 7 through 10). Total glycogen remained unchanged only when a solution contained 0.012 or 0.025 osmol of potassium (Exp. 4 through 6). In all other experiments it was significantly higher or lower than the reference control level.

Addition of phosphate (as sodium phosphate) in the amount of 0.020 osmol to a medium containing 0.055 osmol glucose and 0.025 osmol potassium had no significant effect upon either glycogen fraction (Table II, Exp. 5 and 6). In essence this corroborates results reported on diaphragm and liver(8,10, 13) to the effect that glycogen synthesis in a constant electrolytic environment is not influenced by the addition of phosphate. Consequently, a small amount of phosphate may serve as a buffer in the maintenance of pH of the medium without influencing the experiment.

Oxygen utilization by rat cardiac tissue slices in the presence of potassium is not in-

fluenced particularly until such time as the electrolyte concentration is greatly increased. Respiration does not occur at high osmolar concentrations.

Discussion. The acid-insoluble cardiac glycogen fraction, which is the more stable component, is readily lowered in these *in vitro* experiments regardless of the electrolytic concentration or the osmolarity of the medium. This effect was observed in all experimental series when the comparison was made to the reference control values. It is not improbable that enzymatic degradation may account for at least a portion of this decrease. The glycogenolytic effect becomes more pronounced as the concentration of sodium or potassium is increased or as the osmolarity of the solution is raised. The acid-soluble form of glycogen in rat cardiac tissue is the fraction principally affected. Glucose promotes synthesis of this component(14) which accounts for the increase in total glycogen. These findings were confirmed in this investigation. The optimal medium for synthesis of this glycogen fraction by rat heart tissue slices is one that has an osmolarity of 0.300 and contains either 0.287 or 0.055 osmol of glucose and no electrolytes. A statistical analysis revealed there was no significant difference between solutions containing 0.287 and 0.055 osmol of glucose (Table II, Exp. 1 and 2) and synthesis proceeded equally well in both. In a 0.300 osmolar solution containing 0.010 to 0.100 osmol of sodium and approximately 1% glucose, synthesis of the labile glycogen form also occurs (Table I, Exp. 2 and 4 through 7). However, as sodium concentration is increased to a level of 0.130 osmol or more, or osmolarity of the solution is elevated above that of the normal body fluid, glycogenolysis takes place. It has been shown(15) that this glycogenolysis, observed with tissue slices incubated in a relatively high sodium medium, is due to an increased phosphorylase activity.

Glycogenesis is observed, under the conditions of the potassium experiments, if the electrolyte in question is maintained at a concentration of 0.025 osmol or less. This synthesis is confined to the acid-soluble glycogen fraction. An increase above this level depresses glycogen synthesis or promotes glycogenolysis. Wajzer(16) inferred this when he stated that excess potassium accelerates the decomposition of the lyoglycogen (acid-soluble) in liver or muscle pulp and will involve the desmoglycogen (acid-insoluble) at the same time or shortly thereafter. Potassium also induces greater synthesis of the acid-soluble constituent than does sodium. This was confirmed by a comparison of experiments 4 in each table in which the P value was $<.001$. Cahill and co-workers(15) believe the low level of phosphorylase activity is a factor in the ability of tissue to synthesize glycogen in a potassium medium.

Oxygen consumption in a solution containing glucose and sorbitol is higher than when an electrolyte is added. The general trend of oxygen utilization is toward depression as the concentration of sodium or potassium is increased.

Summary. Rat cardiac tissue slices incubated in a 0.300 osmolar solution containing up to 0.100 osmol sodium or up to 0.025 osmol potassium and 1% glucose, synthesized acid-soluble glycogen. Greater synthesis of this glycogen fraction occurred in a glucose medium if the electrolytes were not present. A further increase of either ion induced glycogenolysis or depressed synthesis of this fraction. The acid-insoluble glycogen fraction was not especially sensitive to the electrolyte content of a solution but glycogenolysis was observed under all experimental conditions and increased as the electrolyte was increased. Oxygen consumption was highest in a 0.300 osmolar solution that contained approximately 1% glucose and 0.010 osmol of sodium and no potassium. As concentration of the electrolytes was increased oxygen utilization decreased. To extract the most information from glycogen synthesis studies the acid-soluble and acid-insoluble glycogen fractions should be investigated.

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Serum Properdin Levels and Cancer Cell Homografts in Man.* (23550)

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Homotransplantation studies with cultivated human cancer cells revealed differences in the ability of healthy persons and patients with advanced cancer to reject the implanted cells(1). The present paper reports parallel differences in serum properdin(2) levels in these same persons. The implantation of cancer cells into normal volunteers elicited a marked local inflammatory reaction and rapid rejection of the implant. In patients with advanced cancer the initial inflammatory reaction was minimal and growth of the implanted cells occurred at almost all sites. Spontaneous regression occurred in some cancer patients but in others growth of the implanted cells was progressive. Attempts have been made to determine the cause of the difference of response of healthy persons and cancer patients to cancer cell implants. None of these studies, except the present investigation, has thus far revealed any gross alterations in cellular or humoral factors that might explain the impairment of homotransplant rejection in the cancer patients (see Discussion). It has been reported elsewhere that treatment of mice and other experimental animals and man with large doses of zymosan or certain polysaccharides derived from bacteria or mammalian tissue cells caused temporary depression of properdin level, while small doses caused transient increases(6-10). It has also been observed that alterations in properdin levels and resistance to infections(11,12), to roentgen irradiation(13), and to hemorrhagic shock(14,15) can be induced by these high molecular weight polysaccharides. Studies in experimental animals also suggest a relationship between serum properdin levels and growth of transplantable tumors. Palm(16) found that rats treated intensively with zymosan, an agent known to influence properdin levels *in vivo,* became temporarily receptive to heterotransplants of the human epidermoid cancer cell line HEp #3. Herbut and Kraemer(17) reported similar results using their human colon adenocarcinoma HR 132 in rats, and postulated that the properdin system might be one of the natural defense factors against neoplasms. Bradner and co-workers (18) found that the transplantable mouse sarcoma S 180 grew faster in mice treated with large doses of zymosan which depressed the properdin levels of these mice, but less well in mice treated with a small dose of zymosan which elevated their properdin levels. Neither these animal studies nor the present clinical

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experiments have demonstrated that properdin has any tumor-inhibitory effect. They merely indicate that conditions which alter serum properdin levels also affect growth of homologous or heterologous transplants. Factors other than properdin may also have been affected by such experimental procedures.

In view of these observations, it seemed of interest to determine whether serum properdin levels differed in the human volunteers who accepted or rejected cancer cell homografts. The present report shows that there are marked differences between the properdin levels of the cancer patients and the normal persons and that a parallelism exists between serum properdin levels of humans and their ability to reject cancer cell homografts.

Methods and materials. The cells (HEp #1, HEp #2, HEp #3, HeLa, HS #1, J-111, Chang's conjunctival cell) implanted into the 2 groups of recipients had been cultivated for long periods (up to 5 years) in tissue culture and/or by passage through cortisone-treated rats or hamsters following their original isolation from human cancer or normal tissues. Although Chang's conjunctival cells were of normal origin they now exhibit the cytologic characteristics of neoplasia(20) and are not considered separately from cells of cancer tissue origin in this paper. The various cell lines were tested in approximately the same proportions in the 2 groups of recipients. The growth of these several types of cells on homotransplantation has been described in a preliminary communication(1) which also gives references to the literature concerning each cell type. *Titration of properdin.* At least one blood specimen from each volunteer was collected for properdin assay on the day of inoculation (day 0) or 14 days thereafter (day 14). From several of the volunteers serial specimens were obtained between day 0 and day 42 or later. After separation from clots, serums were stored for periods up to 6 months at either 5°, –20° or –70°C. Studies in these laboratories have shown that serums could be stored satisfactorily under these conditions. All properdin titers were determined by the zymosan assay(21) employing the same reagents and personnel. Titrations on serums from cancer patients and normals were always run simultaneously. Properdin was titered in at least one serum from each person after the serum had been centrifuged at 35,000 g for 2 hours at 2°C because it has been previously shown(7) that certain serums contain high molecular weight polysaccharide complexes which interfere with the zymosan assay of properdin and which can be removed by ultracentrifugation. This phenomenon was also observed in the present study. An uncentrifuged specimen of each serum was tested simultaneously. Tests were done under code and recorded before correlation with clinical data. Units of properdin/ml of serum are expressed as U. From 1U to 2U is considered a low level of properdin, 3-4U low normal, 5-10U normal, and 12U or greater is considered high.

Results. All 44 serums from the 38 normal volunteers had detectable properdin and gave identical titers before and after centrifugation at 35,000 g. Only 36 of the 53 serums from cancer patients had detectable properdin (11 of the 17 individuals) and 14 of these showed a 1 to 2U drop in titer after centrifugation. The nature and significance of this phenomenon in cancer patients are under investigation. Properdin titers presented hereafter are those determined after centrifugation. However, presentation of the titers of uncentrifuged serums would not materially influence the results.

Serial serum samples from individual persons showed little variation in properdin titer. Among 13 normals from whom serial serums were tested, 5 showed a slightly lower titer 7 to 14 days after implantation than pre-implantation serums. However, no titer went below normal levels and the slight falls were not related to severity of local erythema or induration, or type of cell implanted. In the cancer patients there was no consistent change in properdin titers. One cancer patient had a transient rise from 1U to 4U at a time when an implant was dormant but other cancer patients showed either no change or progressive fall. Since the fluctuations of properdin level during the experimental period were negligible and were unrelated to the phenomenon being studied, all data presented hereafter refer to a single properdin titer in

TABLE I. Distribution of Serum Properdin Titers in Normal Adult Humans and Patients with Advanced Neoplastic Diseases. Entries indicate number of individuals.

Groups	No. of persons	Serum properdin (U/ml) <1	1	2	3–4	5–6	7–8	9–10	11–12
All normals	38			2	4	9	7	13	3
All cancer patients	17	6	3	5	3				
Epidermoid cancer of:									
Bladder, cervix, vulva	5	2	2		1				
Pharynx, larynx, lung	4	1		3					
Adenocarcinoma of:									
Ovary, uterus	2	1		1					
Rectum, sigmoid	2	2							
Lung	1				1				
Sarcomas:									
Melanoma	1		1						
Reticulum cell sa.	2			1	1				

each patient. When serial specimens were tested, the serum collected on or closest to day 14 is recorded.

Comparison of the 2 groups of volunteers (normals and cancer patients) revealed marked differences in properdin levels (Table I). The 38 normal volunteers had a mean properdin level of 7U, and none was below 2U. The 17 cancer patients averaged less than 2U and 6 were < 1U. Overlap between the two groups occurred only at 2 to 4U. Diagnoses are indicated in Table I, but the series is too small to permit any conclusion regarding relationship of type of cancer to properdin levels.

There was also little overlap between the normal and cancer groups in their receptivity to homotransplants. Among the 17 cancer patients, only 2 failed to show nodule formation due to growth of the implanted cells as demonstrated by biopsy. Both of these patients had properdin titers of 4U. Spontaneous regression occurred in 4 patients (properdin titers 4U, 2U, < 1U, < 1U) whose implants were not excised but this started comparatively late (third to fourth week after implantation) and was characterized by mononuclear cell infiltration rather than acute inflammatory reaction. Five cancer patients had persistent or recurrent growth of the implanted cells at day 28 or later. In 3 of these, growth of implants continued throughout the period of the patients' survival (6 to 10 weeks) and one, whose properdin titer was < 1U, had metastases of the homotransplanted cancer cells from the implant site to regional nodes.

In contrast, among the 38 normal recipients the transplants elicited a marked local acute inflammatory reaction which persisted until the material was completely resorbed, usually about 3 weeks. Biopsies showed that only about half of the normal recipients had implanted cells remaining at 14 days, and in these the implanted cells were degenerating and surrounded by an intense inflammatory reaction with polymorphonuclear as well as mononuclear cell infiltration. Only one normal volunteer had demonstrated cancer cells remaining after 14 days. His properdin titer was 2U, the lowest in the normal group. Table II and Fig. 1 and 2 summarize these data and show the parallelism between properdin levels and ability to reject the implanted cells.

Whether considering HEp #3 cells alone (Fig. 1) or all cell types combined (Fig. 2), almost all biopsies taken at 6 to 10 days after implantation contained recognizable cells of

TABLE II. Properdin Levels and Cancer Cell Homografts in Man.

	Reaction to homotransplant (see text for criteria of growth & rejection)			
	Growth with metastases	Growth	Rejection	Serum properdin levels
Cancer patient volunteers	1	14	0	Low
	0	0	2	Normal (3U-10U)
	0	0	0	High
Normal volunteers	0	1	0	Low
	0	0	33	Normal (3U-10U)
	0	0	4	High

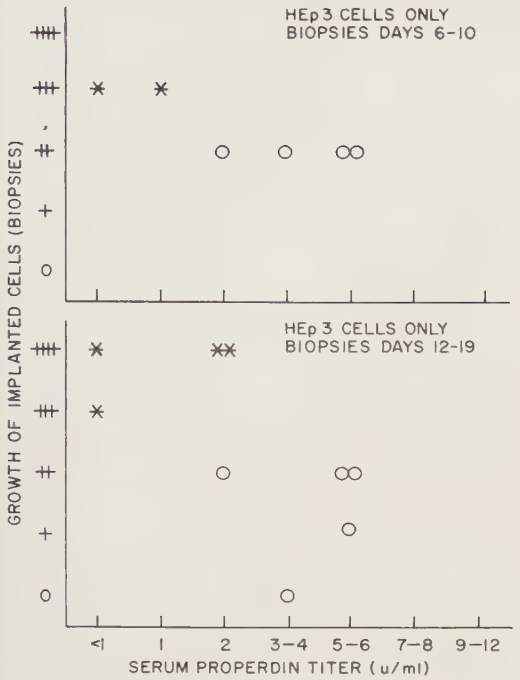

FIG. 1. Relationship between serum properdin and microscopic characteristics of homotransplants of HEp #3 cells at 1 wk (upper graph) and 2 wk (lower graph) after subcut. implantation. Asterisks indicate recipients with advanced cancer. Open circles indicate normals. Biopsies graded as follows: 0, no implanted cells remain, inflammation only; +, few of the implanted cells remain, much inflammation; ++, implanted cells in moderate numbers but inflammatory reaction predominates; +++, considerable numbers of healthy-appearing implanted cells with slight inflammatory reaction, or few cells with no inflammation; ++++, excellent growth of implanted cells without inflammation.

the implanted type. The biopsies from cancer patients showed little or no inflammatory reaction and it was these same patients who had low properdin titers. After another week (bottom half of Figs. 1 and 2) the divergence between the 2 groups was much more marked. Definite propagation of the implanted cells had occurred in the cancer patients with low properdin titers, while regression and marked inflammatory changes were characteristic of the normal recipients with high properdin titers. The number of entries in these graphs does not equal the number of volunteers studied because some individuals were not biopsied within the indicated time periods and some had simultaneous biopsies of 2 implants of different cell types.

Table II simplifies the statement of correlation by making a single evaluation of homotransplant behavior in each *individual.* A recipient is tabulated as supporting "growth" of transplants if some type of implanted cell caused progressive growth of a nodule for at least 2 weeks with histologic demonstration of healthy appearing cells of the implanted type without acute inflammatory reaction or monocytic infiltration. (In 3 cases in which longer follow-up was impossible biopsies taken between days 6 and 9 were accepted as evidence of implant growth because the implanted cells were propagating without evidence of regressive changes.) A recipient is tabulated as showing "rejection" if there was no gross evidence of growth of any implant at any time, or if there was gross and histologic evidence of regression on or before day 14.

Discussion. The foregoing experiments show that the patients with advanced cancer had low properdin titers and that these same patients readily accepted cancer cell homo-

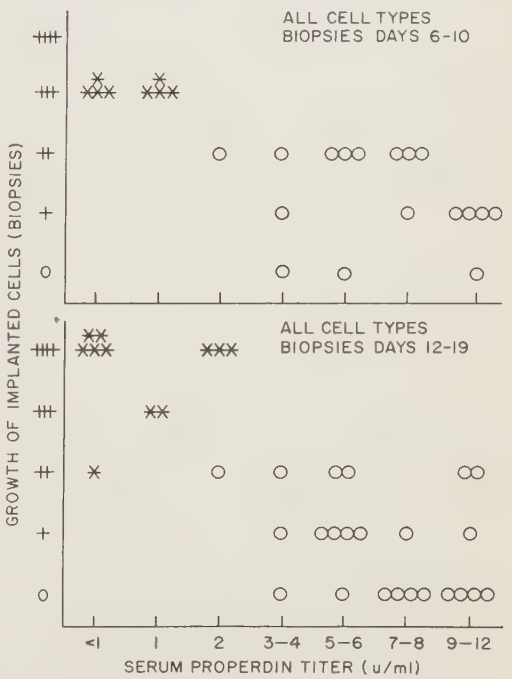

FIG. 2. Relationship between serum properdin and microscopic characteristics of homotransplants of various types of cultivated human cells (HEp #1, HEp #2, HEp #3, HeLa, HS #1, J-111, Chang's conjunctiva) at 1 wk (upper graph) and 2 wk (lower graph) after subcut. implantation. See Fig. 1 for explanation of symbols.

grafts. In addition, there was evidence of a correlation between serum properdin levels and ability to reject homotransplants of cancer cells. There is however no evidence to indicate that this relationship is causal. It must also be emphasized that there is no evidence that the ability to reject homologous cancer cell implants reflects a defense mechanism which controls the occurrence or growth of spontaneous cancer.

Obviously the 2 study groups differed in many respects other than their serum properdin levels. Almost all of the cancer patients had disseminated disease and were cachectic and terminal. The normal volunteers were all male and 6 were Negro. Half of the cancer patients were female and one was Negro. No person in the normal group had any evidence of acute or chronic disease. Median age was 35 years for the normals and 62 for the cancer patients, but there was no relationship between age and properdin levels or growth of implants within either group.

However, none of the cancer patients had received treatment within 3 months of implantation with x-ray, alkylating agents, steroid hormones, ACTH, or metabolic antagonists. None of the patients had an abnormal differential leukocyte count, and only 2 had primary reticuloendothelial system neoplasia. They responded with leucocytosis and fever to bacterial infections. Mobilization of inflammatory cells in response to an irritant was demonstrated in one patient (who had a properdin titer of 1U and whose implanted cells metastasized) after application of croton oil to a scarified area of skin. This area rapidly became red and hot and, as shown by biopsy 6 hours later, was infiltrated by polymorphonuclear leukocytes. All of the cancer patients produced antibodies to viruses administered as experimental therapeutic agents shortly before, during, or after the implant studies. Time of appearance and titer of antibodies seemed normal, but direct comparison of normal persons is not possible. Studies demonstrating antibody production by similar groups of cancer patients have been published(3,4). Complement titers on a similar group of cancer patients showed no major deviation from normal range(5). None of these patients had uremia or gross hepatic dysfunction at the time of these studies. Serum protein partitions on 6 of the cancer patients showed that 4 had hypoproteinemia and hypoalbuminemia and one had a slight hyperglobulinemia, but properdin titers and acceptance of transplants were independent of these protein values.

Since none of the factors which are thought to be related to body defense mechanisms were demonstrably deficient in the cancer patients, properdin remains as the one factor considered to be one of the natural defense mechanisms which thus far has been shown to be deficient in these patients.

There is as yet no explanation for the low properdin levels observed in cancer patients. None of the cancer patients in this study had recently received x-ray or anti-cancer chemotherapy, and infection seems inadequate to explain the properdin depressions. While one patient with a very low properdin titer had a chronic urinary infection (Pseudomonas and mixed coliform bacteria) and another had subcutaneous abscesses (mixed staphylococci, streptococci, and diphtheroids), the others had no obvious bacterial infection. Several patients had experimentally induced virus infections during these studies (West Nile, Mengo, and Semliki Forest viruses) but the serial specimens seemed adequate to exclude viremia, antibody production, or clinical reaction as possible explanations for the low properdin levels. An increased frequency of low properdin titers in cancer patients was also observed by Rottino and Levy(19) and Isliker(22), but not by Hinz(23). The fact that administration to mice of polysaccharides from normal and neoplastic mouse tissues(8) can depress serum properdin levels suggests the possibility that a large mass of cancer tissue may of itself absorb properdin or liberate into the circulation tissue polysaccharides which combine with properdin, thus depressing properdin levels.

Summary. Low levels of serum properdin were found in patients with advanced cancer whose ability to reject cancer cell homografts was defective. Normal properdin levels were observed in normal persons who rejected cancer cell homografts promptly. Investigation

of cellular and humoral defense mechanisms other than properdin has thus far revealed no differences which might explain the impaired rejection of implanted cells by the cancer patients.

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Preparation and Inactivation of Purified Poliovirus: Comparison of Vaccines Derived from Mahoney and Parker Poliovirus.* (23551)

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A great many studies related to preparation of poliomyelitis vaccine have been made, but little work has been done with poliovirus freed from the impurities (nutrient substances, cell components and salts) that accompany the virus in tissue culture fluids.

The advantages inherent in a vaccine derived from purified virus led us to undertake the purification of poliovirus and to study the properties of the purified virus as it became available. With respect to vaccine preparation, we were interested especially in the inactivation of the virus by formaldehyde and the immunogenic properties of the non-infective product.

Methods. Preparation of purified poliovirus. Our studies of the isolation of poliovirus led to a modified version of the Schwerdt and Schaffer procedure(1-3). Details of the isolation have been described(4,5). We have retained the first precipitation step at pH 4.0 to 4.5 with methanol in the presence of celite at 5°C. The filter cake is transferred to a column and eluted with 2% NaCl in 1% phosphate buffer at pH 7.0. This column elution results in a 300-fold increase in virus concentration. The butanol emulsification

* Portions of this paper were presented before 41st annual meeting of Am. Assn. Immunol., Apr. 17, 1957, Chicago, Ill.

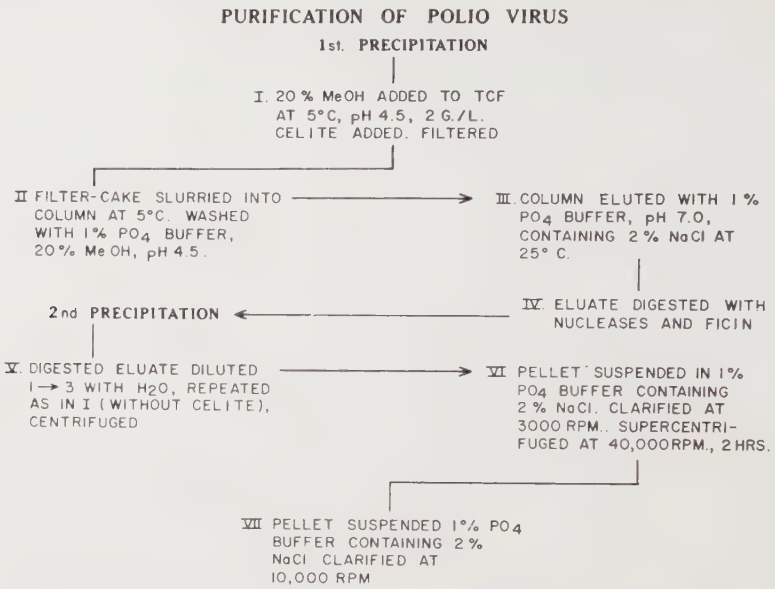

FIG. 1. Detailed flow sheet for the process used to purify large volumes of tissue culture filtrates.

step in the original procedure was abandoned in favor of digestion of non-viral protein with purified soluble ficin, a procedure which in our hands does not result in loss of virus. The digested eluate is reprecipitated and the product ultracentrifuged; pH 7.0 buffer used throughout.

Results. Yields of infectivity and complement fixing material have averaged about 30%. The method is summarized in Fig. 1. Our yields during purification were followed by modified metabolic inhibition test (M.I.T.) (6), plaque count(7) and complement fixation (CF)(8) assays; the relative accuracy and rapidity of the complement fixation method considerably expedited our studies. A comparison of the results of the 3 methods is shown in Table I.

With increasing concentration of virus, measurement of ultraviolet absorption becomes possible, and as purification proceeds the products approach the spectrum of the purified virus, as described by Schwerdt and Schaffer. The final product has a spectrum identical with that published by them(1) (Fig. 2). All 3 types of poliovirus seem to possess identical U.V. spectra.

Discussion. Some characteristics of the purified material may be mentioned. In the original tissue culture fluids (TCF) and also in the final purified products, the ratios of infective to total physical particles (specific infectivity) have ranged from 1 : 2,000 to 1 : 100,000 depending upon the history of the preparations prior to processing. The infective particles thus represent a trace within the total antigenic mass. The weight : infectivity ratios of different preparations vary widely, while the

TABLE I. Comparison of Results Obtained by 3 Assay Methods on Fractions Isolated in the Purification Process.

	M.I.T. titer ($\times 10^6$)	Plaques/ml ($\times 10^6$)	CF	Ratio: Plaques ($\times 10^6$) / CF	Yield (%)
Tissue culture fluid	6.3	13	4	3.3	100
Filtrate	1.3	2.6			
Eluate	710	960	180	5.3	53
U.C.* supernatant	870	1,560	320	4.9	
U.C.* pellet	13,000	17,000	4,800	3.5	35

* U.C. = Ultra-centrifugal.

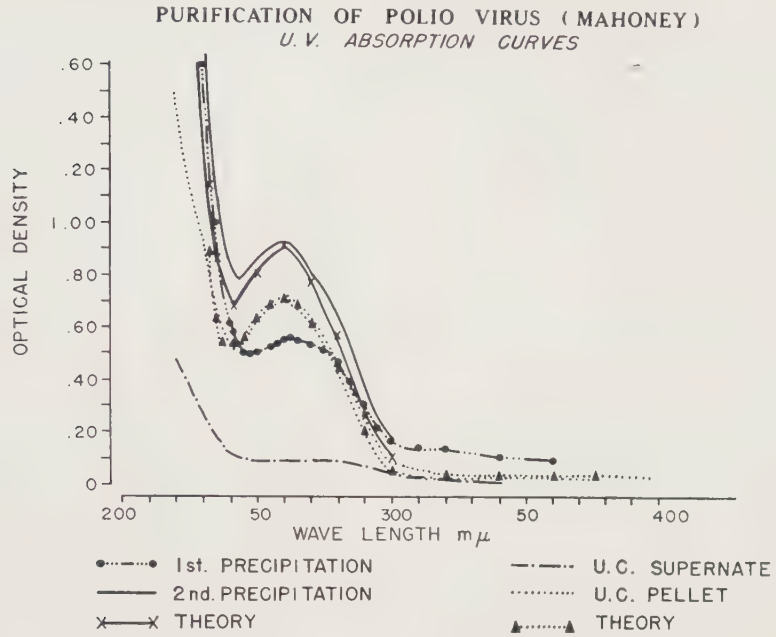

FIG. 2. Ultra-violet absorption spectra obtained on several fractions isolated in the purification process. The theoretical curves were calculated from that published by Schwerdt and Schaffer (1) for concentrations of poliovirus comparable to those prevailing in the isolated fractions.

complement-fixing ability per microgram of purified virus is approximately constant. Inactivation studies therefore reflect the behavior of that small fraction of particles which is infectious, while immunological studies relate to the behavior of the entire mass of particles, infective and non-infective.

Methods. Inactivation of purified poliovirus. The availability of purified virus has enabled us to study the effect of formaldehyde in the extinction of infectivity in such preparations. The conditions chosen were as follows: purified poliovirus was incubated at 37°C in 1% phosphate buffer, pH 7.0, in the presence of 92.5 μg/ml of formaldehyde (1 : 4,000 formalin).

Results. An experimental curve for inactivation of 20 μg/ml of purified Mahoney virus is given in Fig. 3. Examination of data obtained with Type I (Mahoney and Parker strains), Type II (MEF I strain) and Type III (Saukett strain) viruses revealed that the inactivation curves were not only similar but that, except for the difference in intercept depending upon original infectivity, they were identical. Depending upon the quantity and specific infectivity of the virus, curves of parallel slope were observed, indicating, within the precision of the assay used (metabolic inhibition test) (M.I.T.) first order kinetics. This result is to be expected, since formaldehyde is vastly in excess in the inactivation reaction, even when the purified virus is present in a concentration 300 times as great as that found in tissue culture fluids; an excess of one of 2 reactants is the classical condition for the exhibition of pseudo-first order kinetics.

Given these observations, it is meaningful to speak of the half-life of the virus. Under the conditions specified, this half-life is 1.75 hours. A correction may be applied for the inactivation of the virus at 37°C in the absence of formaldehyde, which is a slow process with a half-life of 40 hours. It must be emphasized, however, that the same kinetics cannot be expected to obtain at different pH values or in the presence of formaldehyde-binding impurities such as exist in infective tissue culture filtrates.

The first order inactivation kinetics which we have uniformly obtained are in agreement with the findings of Salk(9) but are not in accord with the recent observations of Timm *et al.*(10), Wesslen *et al.*(11) and Lycke *et*

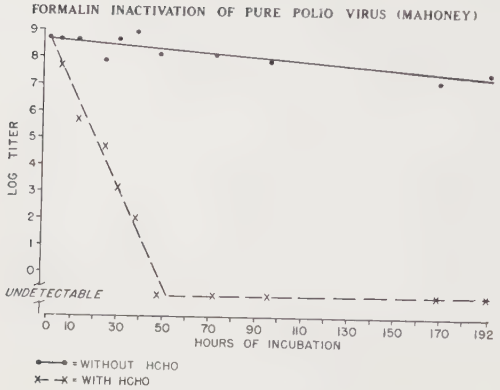

FIG. 3. Graph showing infectivity titers (M.I.T.) obtained during course of formalin inactivation of pure Mahoney virus (20 μg/ml). The inactivation was conducted at 37°C in 1% phosphate buffer (pH 7.0) with 92.5 μg/ml formaldehyde.

al.(12). The conditions used by Wesslen *et al.* and Lycke *et al.* (formaldehyde inactivation at 25°C in the presence of added glycine) are perhaps too different from ours to be usefully compared. The difference between our results and those of Timm *et al.* who observed a disproportionately large fall in infectious titer during the first few hours of inactivation in the case of partially purified virus as well as tissue culture filtrates may, however, merit comment. Apart from the possibility of the effects of differences in technic of sampling and measurement, the nature of the fluids may be decisive. Timm *et al.* note that the degree of deviation from first order kinetics varies from one virus lot to another. Bearing in mind the fact that the infective virus present in ordinary fluids represents a small surviving fraction of a much larger (and perhaps less stable) original population, it is possible that departure from first order kinetics may depend upon a high specific infectivity of the virus preparation. In support of this possibility, examination of a large number of production protocols indicates that inactivation of relatively fresh fluids frequently results in the type of deviation described by Timm *et al.* This effect is infrequent with stored fluids which, in general, show some loss of infectious titer and in which the specific infectivity has thus been lowered. In our purified virus preparations the specific infectivity has, in general, been low.

Methods. Comparison of purified Mahoney and Parker poliovirus. The immunogenic potency of some of our preparations has been determined. Our interest centered initially on the Type I component. This component is thought to be a poor antigen; its use therefore should rigorously test the capacity of a purified vaccine to immunize. The availability of purified vaccine should also permit definitive studies of the inherent antigenicity of different strains; a comparison of the Parker and Mahoney strains was made as described below.

A single lot of monkey kidney monolayer cultures was prepared. Half the flasks were seeded with Mahoney and half with Parker virus.† From the infected fluids, purified virus of each type was isolated. The titer of the Mahoney fluid was $10^{6.5}$ $TCID_{50}$/ml; that of the Parker fluid was $10^{6.3}$ $TCID_{50}$/ml. The complement fixing titer of the Parker fluid was twice that of Mahoney, and after purification, twice the quantity of purified Parker virus was isolated in comparison with Mahoney.

The purified viruses, at a concentration of 20 μg/ml, were inactivated with formaldehyde under the conditions described above. Suitable dilutions of the vaccines and the viruses from which each was derived were tested for potency in chicks.‡ Two doses were given, the first at 7 days of age and the second at 21 days of age. At 28 days, blood was obtained by cardiac puncture and antibody titers were determined by the metabolic inhibition test. Approximately 15 chicks received each dilution of vaccine and the geometric mean titers of each group of chicks were determined. Statistical analysis indicated that the log-dose response curves were linear and parallel within the limits of experimental error. The best fitting straight lines

† The authors are indebted to Mr. Robert Rotundo and associates in the Biological Production Laboratories, Merck Sharp & Dohme Division for supplying these materials.

‡ The authors gratefully acknowledge the cooperation of Dr. J. E. Prier and his co-workers of the Biological Development Laboratories, Merck Sharp & Dohme Division, in conducting the chick test.

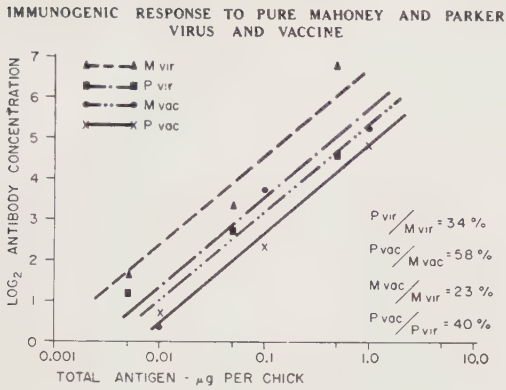

FIG. 4. Dose response curves based on data obtained in chick potency test.

were calculated by the method of least mean squares assuming a common slope.§ The log-dose response curves are shown in Fig. 4 where the logarithm (base 2) of the antibody titer is plotted against the logarithm (base 10) of the dose.

Results. The vaccine derived from Parker virus was 58% as potent as that derived from Mahoney; this difference was found not to be significant. The parent infective viruses differed significantly; Parker virus was 34% as potent as Mahoney. In this connection the 2-fold yield of Parker with respect to Mahoney should be considered; if the potency were to be calculated taking into account the original concentrations of the 2 viruses in the tissue culture fluids, or the yields of purified virus isolated, these differences would disappear. The vaccines were less potent than the viruses from which each was derived. Parker vaccine was 40% and Mahoney vaccine 23% as potent as the parent viruses from which they were prepared. These differences, although small in relation to the precision of the chick potency assay, were found to be significant.

Summary. 1) Purified virus was prepared from tissue culture fluids containing Type I (Mahoney and Parker strains), Type II (MEF I strain) and Type III (Saukett strain) poliovirus. 2) The purified viruses were successfully inactivated with formaldehyde and the inactivation kinetics studied. 3) Purified Type I (Mahoney and Parker strains) viruses and the vaccines derived from them were shown to be immunogenically potent in chicks. 4) No statistical difference was found in the immunogenic potency of purified Parker and Mahoney vaccines in the chick. 5) Purified infective Mahoney virus was found to be significantly more potent than Parker virus. 6) Vaccines derived from purified Mahoney and Parker viruses were significantly less potent than the parent viruses.

§ We wish to express our thanks to Mr. Joseph L. Ciminera, Biometrician, for evaluating the results of the chick test.

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Effects of Acetazoleamide, Chlorothiazide, and Dichlorphenamide on Electrolyte Excretion in the Alligator.* (23552)

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Although the theory that potassium and hydrogen compete for renal tubular excretion (1) is of considerable value in explaining some of the functions of carbonic anhydrase in the mammal, it is inadequate to explain the action of this enzyme in the alligator. Inhibition of carbonic anhydrase by acetazoleamide in the mammal leads to acidemia with an increased excretion of both bicarbonate and sodium. In the alligator, inhibition promotes an alkalemia as a result of excessive chloride excretion with little change in sodium loss(2). Diamox (acetazoleamide) enhances the excretion of K in both mammals(1,3) and alligators(4) in spite of the fact that it decreases the urinary H ion in the mammal and increases it in the alligator.

Several experiments were designed to determine if increased K output is directly related to the increased urinary hydrogen ion excretion. It was also of interest to see if prolonged administration of Diamox to alligators could either induce a refractory response to the drug or if the continued daily loss of plasma chloride would result in a gradual decrease in Cl excretion. In the course of these investigations 2 new carbonic anhydrase inhibitors(5,6), Diuril (chlorothiazide), and DCPA (dichlorphenamide) were made available. Their effects on the alligator are reported.

Methods. About 150 different 2 kg alligators were used in these experiments. The animals were fasted for 4 days before any experiment to minimize the effect of a rather considerable "alkaline tide" and were maintained in this condition throughout any given experiment. To ensure a relatively uniform degree of hydration they were kept in a tank of water for 24 hours before the beginning of each experiment. The chemical methods employed for the analysis of the samples have been described(2,7). In the short term experiments urines were collected in an ice bath and frozen immediately in a deepfreeze until the time for analysis. Blood was drawn by intracardiac puncture, heparinized, centrifuged in the cold, and the plasma separated and kept frozen until it could be analyzed. The experiments were done at a temperature of 28° ± 2°C and at an average relative humidity of 60%.

Results. To determine the effect of prolonged injections of Diamox (50 mg/kg), 12 alligators were placed in metabolism cages where they received the drug in aqueous suspension every other day for 30 days. The daily urine volume was measured and one fresh catheterized sample was obtained each day for estimation of the volatile NH_3 and CO_2.

It was desirable to obtain data on carbonic anhydrase inhibition in both "wet" and "dry" alligators because of the fact that there is an increase in excretion of both NH_3 and CO_2 following hydration and a decrease in these ions during dehydration(7). Therefore 6 of the animals were allowed free access to distilled water for a period of one hour each day in order to maintain a moderate degree of dehydration and 6 were overhydrated by the injection of enough water each day to maintain the original weight of the animals. Fig. 1 shows the total meq/kg of various urinary constituents excreted over the 30-day period. In the groups given Diamox there was a considerable increase in K and Cl excretion and no important change in Na in either the "wet" or "dry" experiments. Although an increase in K excretion occurred along with an increase in H ion excretion, there was no evidence of a direct relationship between degree of decrease in urine pH and amount of K in a given sample. While the urine pH increased

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† Public Health Service Research Fellow of Nat. Heart Inst.

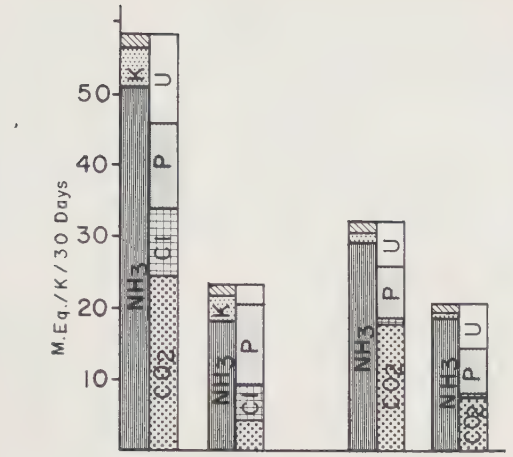

FIG. 1. Effect of intraper. inj. of 50 mg/kg of Diamox every other day for 30 days on urine output. Each bar graph represents total amount excreted for 30 days. Two separate experiments are represented, one over-hydrated and one slightly dehydrated. Six alligators were used in each experiment. Cross-hatched area above the K represents Na.

somewhat in the last days of the experiments, the elevated Cl excretion persisted for the full 30 days in spite of decreased plasma Cl and an increased plasma pH. It was concluded that there was no clear cut evidence of development of a tolerance to the drug and that the chloruretic action of Diamox was largely independent of minor changes in plasma composition.

Blood was drawn at the beginning, at 15 days, and at 30 days of the long term experiment on Diamox. There were no significant changes in K or indeed in any of the ions in the plasma other than Cl and CO_2. By the 15th day there was a 11 meq/l fall in plasma Cl and a reciprocal rise of 11 meq/l in plasma bicarbonate. The 50% increase in bicarbonate is evidence of an alkalemia which is about as marked as the acidemia reported to be the result of repeated injections of Diamox to mammals.

After Diuril and DCPA were made available it was decided to determine if the observed effects were unique for inhibition of carbonic anhydrase by Diamox or if they were the same regardless of the means of inhibition. A pilot experiment was performed by injecting aqueous suspensions of each drug in doses of 0.1, 1, 10, 25, and 50 mg/kg to get a crude estimate of the minimal effective dose. Using the results from the preliminary experiment groups of alligators were given 1 mg/kg (the marginal dose) and other groups received 50 mg/kg (the dose for maximal effect).

The experiments were repeated on large numbers of alligators until the average daily urine flow for any one group varied by no more than 20% from the average of the other groups. Although control of the urine volumes reduced the random variation in total electrolyte excretion, it was not always possible to reproduce the same osmotic pressures. If one uses the daily output of salts as an index of the activity of a compound, a high osmotic pressure is associated with a high excretion of Na, K, Cl, CO_2 and NH_3, which tends to exaggerate the effects. If, however, the Na and K are compared with the NH_3, and the Cl is compared with the CO_2, the effect of any particular compound on the excretion pattern is immediately apparent. A graph was therefore employed (Fig. 2) which illustrates the *ratio* of Na and K to NH_3 and of Cl to CO_2. It is evident that any increase in Na and K must be at the expense of NH_3 and that any increase in Cl must be at the expense of CO_2. The first 3 columns show the maximum range of variation which we have found for the 40 controls whose average appears in column II. It would be unwise to attach much significance to any change fall-

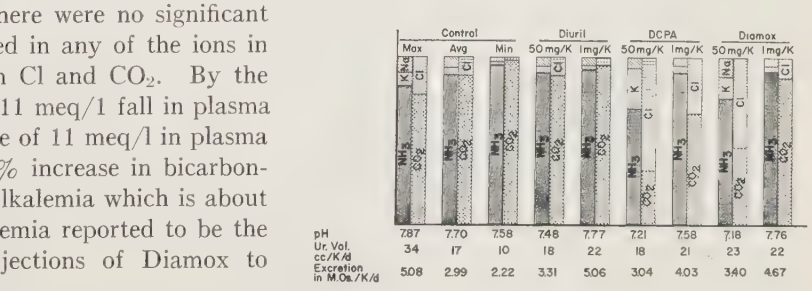

FIG. 2. Ratio of Na and K to NH_3 and of Cl to CO_2 in alligator urine. Each bar graph represents the avg for a total collection of 3 consecutive days. Each alligator received 13 cc of water/kg. There were 40 alligators in the control group and 15 or more in each of the other groups.

ing within the range of variation of the controls.

Diamox and DCPA were both effective in the larger dose in inhibiting CO_2 output and in producing a chloruresis whereas Diuril proved to be inactive. The inactivity of Diuril is in keeping with the observation by Moyer *et al.*(8) that its saluretic properties in mammals were not associated with carbonic anhydrase inhibition. Diamox is certainly less active than DCPA and at a level of 1 mg/kg the significance of the change in Cl in the Diamox group is highly questionable. One surprising feature of the action of DCPA is its ability to cause a much greater increase in K excretion than that found for Diamox. In the larger doses, the greatest K loss in any alligator receiving Diamox was below that found for the least change in any alligator injected with DCPA. On the other hand a slight and possibly significant rise in Na follows the injection of large doses of Diamox but not DCPA. Whereas 1 mg/kg of DCPA was enough to cause a 3-fold increase in Cl excretion, it was insufficient to change the K excretion which makes it appear that depression of CO_2 production and enhancement of Cl excretion is not necessarily associated directly with K loss.

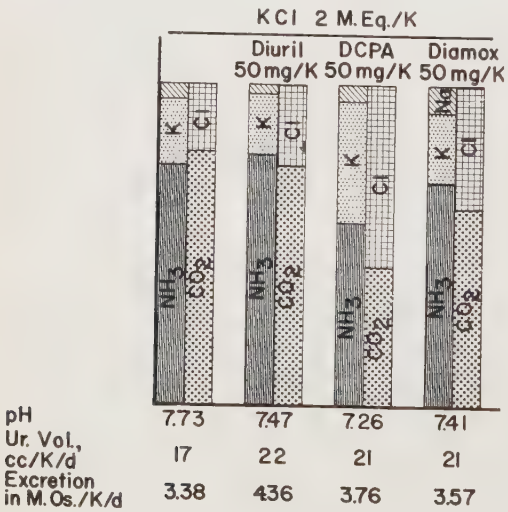

FIG. 3. Effect of Diamox, Diuril and DCPA on urinary ratio of Na and K to NH_3 and Cl to CO_2 in alligators primed with 13 cc of isotonic KCl/kg. There were 15 alligators in each group. Each bar graph represents the avg for a total collection of 3 consecutive days.

On the theory that all 3 compounds could influence K excretion if the plasma level was elevated, the high dose experiments were repeated on alligators primed with 2 meq/kg of KCl. The results are shown in Fig. 3. Increasing the plasma K level did not alter the relative effectiveness of the three compounds. Diuril was still inactive and DCPA was the most active as before.

Discussion. The decrease in CO_2 production caused by Diamox and DCPA is most probably the result of a direct inhibitory effect of these compounds on the tubular production of CO_2 by carbonic anhydrase. A decrease in renal CO_2 production in the alligator inevitably produces an increase in Cl excretion. The existence of a direct relationship between K excretion and carbonic anhydrase inhibition in the alligator is by no means certain. Neither is there any evidence of a positive correlation between the changes in urinary H^+ concentration and the quantity of K appearing in the urine. In view of the above it would appear that the differences in the effects of Diamox and DCPA are an indication that the actions of carbonic anhydrase are more complex than was suspected, or that the individual effects are not necessarily associated with carbonic anhydrase inhibition but with unknown reactions associated with differences in their respective molecular structures.

That something more than simple carbonic anhydrase inhibition is involved is illustrated in Table I which compares the effects of the 3 compounds on dogs(9,10) and alligators. It would appear that the knowledge that a compound will inhibit the carbonic anhydrase in horse erythrocytes provides little help in predicting the action of the compound on intact animals.

Summary. Prolonged administration of acetazoleamide to alligators leads to the excretion of considerable amounts of chloride which produces an alkalemia. A continued K excretion also occurs which has little effect on the plasma level, presumably due to a removal of K from the cells in an effort to compensate for the loss. No evidence of the development of a refractory state to acetazole-

TABLE I. Comparison of Effects of 3 Different Carbonic Anhydrase Inhibitors on Urine Electrolytes of the Dog and the Alligator.

Compound	Dog					Alligator				
	Na	Cl	K	CO_2	H	Na	Cl	K	CO_2	H
Chlorothiazide	++	++	+	+	—	0	+?	0	—?	+?
Dichlorphenamide	++	+	+	+	—	0	+++	++	——	+
Acetazoleamide	++	0?	++	++	——	+?	+++	+	——	+

Direction of the change is indicated as follows: +++, great increase; +, increase; ——, great decrease; —, decrease; 0, little or no change.

amide after 30 days of injections was noted nor was there any sign that the decreased plasma Cl prevented the excretion of Cl. The degree of K loss was not directly correlated with hydrogen ion excretion. Two new carbonic anhydrase inhibitors, chlorothiazide and dichlorphenamide were tested. Dichlorphenamide was more active in promoting K and Cl loss at low dose levels than acetazoleamide. Chlorothiazide showed no significant activity. Since the effects noted after the inhibition of carbonic anhydrase by acetazoleamide and dichlorphenamide are not the same, it is probable that something more than simple inhibition is involved.

The authors wish to thank Lederle Laboratories Division, American Cyanamid Co. for generous supply of Diamox and Merck Sharp and Dohme, for a supply of Diuril and dichlorphenamide.

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Blood Epinephrine Levels and Automatic Reinfusion of Blood During Hemorrhagic Shock in Dogs.* (23553)

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Vasoconstriction is a prominent feature of the early stages of hemorrhagic shock in dogs. Most authorities agree that this is initially a protective mechanism mediated through the carotid sinus to maintain a blood supply to vital organs. However, if this vasoconstriction is of adequate severity and duration it is possible for the ischemia to produce irreversible tissue damage(1). This concept is supported by the fact that several investigators have produced shock by the intravenous infusion of epinephrine(2), and that adrenergic blocking agents protect dogs against hemorrhagic shock(3). Various procedures for producing hemorrhagic shock in dogs have been standardized by Wiggers(4) and other investigators. When dogs are bled to a mean arterial pressure in the range of 40-60 mm Hg by means of an arterial pressure compensator (5) spontaneous reinfusion will commence in about one hour and continue until the animal

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succumbs. Glasser and Page(6) associated this reinfusion with irreversibility in hemorrhagic shock. Beck and Dontas(7) have shown by direct nerve potential measurements that sympathetic activity was greatly elevated during the initial stages of hemorrhagic hypotension and then decreased as automatic reinfusion progressed. Watts(8) has recently shown that high concentrations of epinephrine appear in the peripheral blood of dogs during acute hemorrhagic hypotension and there was evidence that this epinephrine level decreased in some animals after about one hour. The automatic reinfusion of blood at a constant arterial pressure is probably due to an increased vascular space resulting from decreased vasomotor tone. This could be due to failure of the sympathetic vasoconstrictor mechanism, decrease in the concentration of humoral vasoconstrictor substances, or damage to vascular muscle cells. In the present experiments, peripheral blood epinephrine levels have been followed in dogs during the initial stages of hemorrhagic shock and during the automatic reinfusion at a mean arterial pressure of 40 mm Hg to determine whether or not this vasoconstrictor material was disappearing from the blood during the stage of reinfusion.

Methods. Twelve healthy mongrel dogs with an average weight of 11.6 kg (8.4-15.0) were anesthetized with 30 mg/kg sodium pentobarbital injected intraperitoneally 30 minutes before hemorrhage. This amount of pentobarbital was usually sufficient to maintain anesthesia, but small supplementary doses were used as needed to keep the animal quiet. A polyethylene catheter (0.070 in. internal diameter) was passed through a femoral artery as far as the aorta. Heparin (3 mg/kg) was given intravenously to prevent clotting of blood during hemorrhage and collection of samples. Control blood samples were drawn and the dog was allowed to hemorrhage into a polyethylene reservoir. Level of blood in the reservoir was maintained at 40 mm Hg until death of the dog. The blood was agitated with a magnetic stirrer driving a small plug covered with polyethylene. The use of non-wettable polyethylene catheters

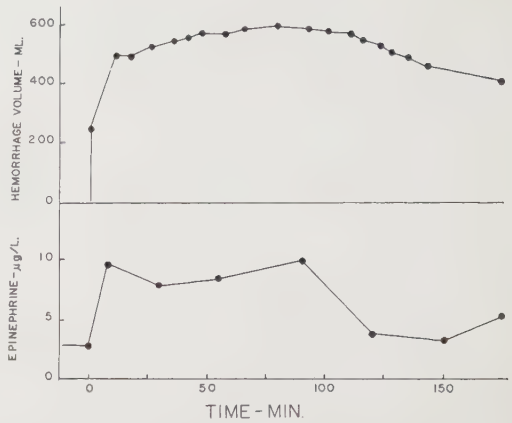

FIG. 1. Hemorrhage vol and blood epinephrine levels in 10.6 kg male dog while mean arterial blood pressure was held at 40 mm Hg. Blood samples were drawn from the femoral artery and epinephrine values are given as the base.

and containers minimized hemolysis. Temperature of the blood was maintained at 38° C. *Epinephrine levels* in arterial blood were determined at 6 to 30 minute intervals beginning with control samples taken before hemorrhage. With minor modifications the method of Gaddum and Lembeck(9), employing the rat uterus was used for the bioassay for epinephrine. Modified Ringer solution was used as the perfusion fluid. Carbachol (1 mg/l) was used to stimulate the uterus. Lysergic acid diethylamide (5 µg/l) was added to the wash solution to prevent stimulation of the uterus by serotonin in the blood samples(10). Two to 3 ml blood samples were drawn from the femoral artery into iced polyethylene tubes and assayed within 30 seconds since extensive experience with the method has shown that epinephrine disappears very rapidly from shed blood. Under these conditions from 93 to 99% of epinephrine added to blood samples can be recovered. Hemorrhage volume, respiration, and heart rate were recorded at the time each blood sample was obtained. Tests for significance were made by Fisher's(11) method for small samples using the "t" test to determine probability.

Results. Fig. 1 is a typical experiment showing hemorrhage volume and blood epinephrine levels during hemorrhagic hypotension at 40 mm Hg with automatic reinfusion

TABLE I. Epinephrine Levels in Peripheral Arterial Blood of Dogs during Hemorrhagic Hypotension at 40 mm Hg with Automatic Reinfusion.

	Epinephrine—μg/l, as the base				Hemorrhage vol—ml/kg	
	Control	Max hemorrhage	Early reinfusion (first 10%)	Late reinfusion (10% to death)	Max	At death
	.9	17.3	9.5	5.8	51.2	31.0
	0 *	4.0	6.7	8.8	45.0	0
	3.4	7.7	6.0	4.1	54.8	43.3
	0	5.2	8.7	5.1	32.3	10.1
	0	9.3	5.9	4.9	46.7	26.0
	0	14.2	6.4	1.2	40.5	24.8
	2.9	9.7	5.3	3.4	40.6	24.5
	0	10.8	4.9	4.5	70.6	44.1
	0	7.5	6.8	6.1	34.7	26.0
	0	4.9	3.8	2.5	50.4	
	0	21.0	6.5	4.3	45.5	
	0	38.0	22.2	11.6	48.1	
Mean ± S.E.	.6 ± .33	12.5 ± 2.75	7.7 ± 1.39	5.2 ± .80	46.7 ± 2.72	25.5 ± 4.7

* 0 indicates epinephrine content of blood was less than sensitivity of the method which is about 1 μg/liter.

in a 10.6 kg male dog. Automatic reinfusion began 90 minutes after the start of hemorrhage and continued until the animal died at 178 minutes. The blood epinephrine levels paralleled the hemorrhage volume. The maximum epinephrine concentration was found soon after hemorrhage was started and remained at this elevated level until about the time automatic reinfusion began. As the reinfusion continued the epinephrine level decreased and was near the minimum level observed at the time of death.

Table I summarizes the data from similar experiments on 12 dogs. Since the time to reach maximum hemorrhage, for automatic reinfusion, and for death, was quite variable, the blood epinephrine levels are given for the control period, the time to reach maximum hemorrhage and for periods of early and late reinfusion. Early reinfusion was arbitrarily taken as the time required for reinfusion of 10% of the hemorrhaged blood; late reinfusion as the time from the point of 10% reinfusion until death. The average maximum hemorrhage of 46.7 ml/kg was reached in 89 minutes (range, 60-135). Automatic reinfusion commenced at this point and 10% of the maximum hemorrhaged volume had returned to the circulation at the end of 127 minutes (range, 90-210). The reinfusion continued and the animals died after an average elapsed time of 232 minutes (range, 140-395) when average hemorrhage volume was 25.5 ml/kg.

Epinephrine concentration in the peripheral blood increased from a control level of less than one μg/l, the minimal concentration of epinephrine that could be detected with the rat uteri used, to a level of 12.5 ± 2.7 μg/l during the period of maximum hemorrhage. This is a significant increase. This epinephrine concentration reached a peak at approximately the same time as the hemorrhage volume. The blood epinephrine level then decreased during the period of early reinfusion to an average of 7.7 μg/l, and further decreased to an average of 5.2 μg/l, during late reinfusion. These epinephrine concentrations during both the early and late stages of automatic reinfusion are significantly less than the level during the period of maximum hemorrhage. The heart and respiratory rates were elevated above control values throughout the period of hemorrhage except for a short interval when death was imminent and both rates dropped precipitously. Respiratory exchange was good in all animals except for the last few minutes before death.

Discussion. These experiments have shown that epinephrine concentration in the circulating blood is significantly higher during hemorrhagic hypotension than under normal conditions. The method of bioassay used was not sufficiently sensitive to detect routinely epinephrine in the control blood samples. Thus

epinephrine levels in normal blood could only be determined to be less than the minimum sensitivity of the method, *i.e.*, less than about one μg/l. This observation accords with the results of Holzbauer and Vogt(12) showing that in a highly purified extract that the plasma epinephrine concentration in a dog under normal conditions was between 0.04 and 0.25 μg/l. The rise in the epinephrine content of the blood during hemorrhage and the maintenance of this high concentration indicate that epinephrine release may not only be one of the compensatory mechanisms effective in shock, but may even be the principal one. The average peak of 12.5 μg/l, of epinephrine represents at least a 10-fold increase over normal physiological levels and could account for the intense vasoconstriction, decreased peripheral blood flow in many tissues, and cardiac effects which are typical of hemorrhagic shock in dogs(4).

The impending failure of the cardiovascular system as indicated by the automatic reinfusion of blood was accompanied by a lowering of the epinephrine content of the peripheral blood. The decrease in blood epinephrine between peak hemorrhage and death is statistically significant, but it should be indicated that throughout the period of hemorrhage and at the time of death, blood epinephrine levels are well above the control level. Although the physiological significance of the maximum blood epinephrine levels observed in these experiments has not yet been established, it is very likely that this circulating epinephrine does contribute to the intense vasoconstriction and tissue ischemia characteristic of the earlier stages of hemorrhagic shock. Since the concentrations observed throughout the period of hypotension are well in excess of normal physiological levels, it appears that the administration of additional epinephrine would not be beneficial, whereas adrenergic blocking agents could be expected to alleviate some of the suspected deleterious effects of this excess circulating epinephrine.

Summary. The epinephrine content of peripheral blood of 12 dogs was determined during hemorrhagic hypotension with automatic reinfusion of blood at 40 mm/Hg. The epinephrine content increased from a control value of less than one μg/l to a maximum of 12.5 μg/l which occurred during the period of maximum hemorrhage. This epinephrine level decreased during automatic reinfusion of the blood at 40 mm Hg and reached a level of 5.2 μg/l during the later period of the reinfusion before the dogs died. It is possible that this increased concentration of epinephrine in the circulation is at least partially responsible for the intense vasoconstriction, decreased peripheral blood flow in certain tissues, and detrimental tissue ischemia, typical of hemorrhagic shock in dogs.

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Blood Cell Changes Following ACTH Injection in the Chick.* (23554)

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Criteria of pituitary-adrenocortical function in the mammal in response to application of stressors or ACTH injection, in general have been found not to be adequate following similar treatments in birds. Adrenal hypertrophy has been reported not to occur in the chick following administration of various noxious agents(1) and to occur following chronic administration of ACTH (extract) or epinephrine(1,2). ACTH, epinephrine or cold did not produce an acute decrease (within 1-4 hours) in adrenal ascorbic acid content in the chick, duck or quail(2-5). Slover(6) demonstrated a decrease in adrenal ascorbic acid 12 hours following injection of ACTH in White Leghorn cockerels. Stamler *et al.*(7) failed to obtain an eosinopenia in the chick following chronic ACTH treatment. Elton and Zarrow(5) reported no depletion of adrenal cholesterol following ACTH injection; Slover (6) found a decrease in adrenal cholesterol 6 hours after ACTH injection in both male and female chicks, however repetition failed to substantiate this unpublished result.

Lack of suitable physiologic indicators of stress in the bird has prevented evaluation of the effects of stressors in the bird. The purposes of this investigation were: (a) to study the changes in blood cell counts of the chick following a single injection of ACTH and (b) to assess the possibility of using any resulting changes as possible indicators of stress (assuming that endogenous liberation of ACTH is a requisite event in the response of the bird to stressors).

Procedure. White Leghorn cockerels were obtained from a commercial hatchery on the day of hatching and maintained on Purina *Startena* and water *ad lib.* in brooder batteries. At 1-2 weeks of age, birds were injected with ACTH (ACTHAR, lyophilized, Armour) intraperitoneally; uninjected birds or water-injected (0.1 ml) birds of like age served as controls since in preliminary work it was found that neither water (0.1 ml) nor a dry needle puncture produced any significant changes in blood cell counts. At a specific time following injection of the hormone, intracardial samples of blood (1 ml or more) were drawn in a heparinized syringe from both treated and control birds. *Hematocrits* were obtained by centrifuging blood samples in Van Allen pipettes until constant readings were obtained. Direct acidophil counts were made in a standard hemocytometer at least 24 hours following dilution (200X) with Wiseman's staining solution(9); each blood sample is represented in the calculations by the mean of 4 counts. Differential white cell counts were made from smears stained with Wright's stain; a total of 200 cells was counted from each smear. Total white cell counts and absolute numbers of each kind of leucocyte were obtained by appropriate calculation from the direct acidophil and differential counts.

Results. Results of determinations on blood from cockerels 3, 6, 12 and 24 hours following intraperitoneal injections of ACTH are shown in Table I. Injection of ACTH was followed by an increase in total leucocytes at the 4 time periods following treatment. This leucocytosis was greatest during the first 6 hours and decreased in extent at the 12 and 24 hour intervals.

Changes in absolute numbers of eosinophils, basophils and monocytes were neither great in extent in most instances nor consistent, perhaps due to the small numbers of cells involved and therefore the relatively greater error involved in calculation. Furthermore, the changes in absolute numbers of these 3 types of cells in the treated birds were not beyond the range of variation found in the control groups. A marked heterophilia (neutrophilia) was obtained at all periods sampled except for one trial (24 hours). The increase in heterophils was greater at 3 and 6 hours than at 12 and 24 hours. A slight lymphocy-

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TABLE I. Blood Cell Determinations on Control and ACTH-Treated (1 IU) Birds.

Group	Treatment	No. birds	Acidophil count†	Total WBC‡	Basophils	Eosinophils	Heterophils	Lymphocytes	Monocytes	RBC§	Hematocrit
1	None	13	8.67 ± 5.38	11,665	210	315	1,610	9,355	175	2.064	33.7
	ACTH + 3 hr	14	19.10 ± 9.59	15,475	186	309	3,931	10,832	217	2.244	31.3
2	None	15	9.25 ± 5.16	14,260	242	171	1,882	11,751	214	2.078	27.4
	ACTH + 3 hr	14	24.34 ±17.67	18,568	278	56	5,348	12,663	223	2.009	25.9
3	None	16	5.84 ± 3.55	6,970	153	98	1,199	5,416	104	1.986	
	ACTH + 6 hr	15	19.67 ±10.38	14,270	242	143	4,224	9,390	271	1.894	
4	None	15	9.36 ± 4.72	11,806	248	59	2,019	9,362	118	2.149	31.2
	ACTH + 6 hr	13	36.69 ±23.87	18,201	182	27	8,118	9,774	100	1.969	28.0
5	None*										
	ACTH + 12 hr	15	13.88 ± 8.26	12,131	170	194	2,887	8,638	242	1.989	
6	None	25	5.82 ± 3.22	10,157	179	329	963	8,552	134		
	ACTH + 12 hr	19	10.92 ± 5.29	13,125	228	525	1,899	10,204	269		
7	None*										
	ACTH + 24 hr	16	9.39 ± 9.74	10,233	187	89	1,995	7,757	205	2.033	
8	None	16	10.73 ± 5.35	14,178	354	241	2,141	11,144	298	1.928	
	ACTH + 24 hr	15	7.02 ± 2.75	11,717	117	105	1,453	9,749	293	1.899	
9	None	12	11.38 ± 4.56								
	Needle	11	12.98 ± 5.44								
	H_2O + 6 hr	12	10.68 ± 4.82								
	H_2O + 12 hr	12	8.54 ± 4.37								
	H_2O + 12 hr	12	13.85 ± 7.69								

* All birds in this group were processed at the same time as group 3; those inj. with ACTH are to be compared with the controls of group 3.

† Count = No. of acidophils/0.045 mm^3 blood ± stand. dev.

‡ WBC and leucocytes expressed as cells/mm^3 blood.

§ RBC expressed as millions/mm^3 blood.

tosis was obtained in 6 of the 8 reported trials; the magnitude of this increase was greatest at 3 hours and decreased thereafter. From the above results it is evident that the observed leucocytosis was due primarily to the heterophilia and to a lesser extent to the lymphocytosis.

The above absolute numbers of leucocytes are based on acidophil counts which, in the chicken, are comprised of both eosinophils and heterophils (neutrophils). Injection of ACTH resulted in a statistically significant ($P = < .005$) increase in acidolphils throughout the first 12 hour period. This acidophilia is evidently due to the marked and consistent heterophilia rather than to an eosinophilia.

Examination of the diffential counts on which the absolute numbers of cells in Table I are based, indicates a consistent relative increase in heterophils and a decrease in lymphocytes following injection of ACTH.

Treatment with ACTH was followed in the 3 instances where determined by a lowered hematocrit of 5.5, 7.2 and 10.3% of the respective control values. The RBC counts indicate no great or consistent change in the treated birds, but were slightly lower in 4 of 6 determinations. This decrease is consistent with the lowered hematocrit.

Neither injection of distilled water (0.1 ml) nor puncture with a dry needle produced a significant change in acidophilia 6 or 12 hours following treatment (see group 9, Table I). These results indicate that the acidophilia obtained following injection of ACTH was not due merely to the mechanics of injection.

To determine if the degree of acidophilia was proportional to dose, graded doses of ACTH were administered to 2 series of birds. Dose-response curves based on data obtained 6 and 12 hours following injection are shown in Fig. 1. Statistical analysis indicated that there is a highly significant (beyond the 1% level of probability) linear relation between

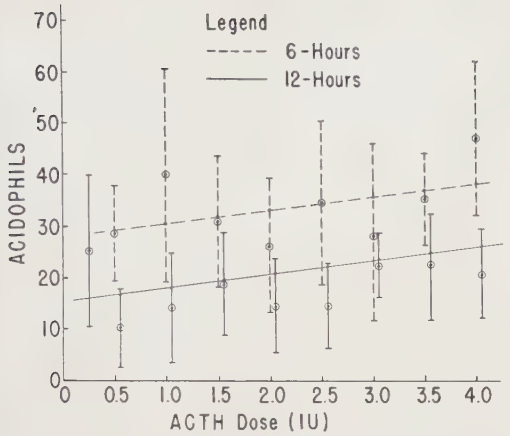

FIG. 1. Response of acidophils (number/.045 mm³ blood) to graded doses of ACTH (IU). Each point represents the mean of 12 birds; vertical lines represent ± stand. dev. of means.

dose and magnitude of response in each case. In the 6-hour trial, $Y = 27.85 + 2.65\ X$; in the 12-hour trial, $Y = 15.55 + 2.65\ X$. It will be noted that the acidophilia was uniformly greater at the 6-hour period than at the 12-hour period. Coefficients of variation of the acidophilia were: 44.67% for the 6-hour series and 52.23% for the 12-hour series; refinement of procedure could possibly reduce this high value if it could be shown that its sources were not predominantly attributable to the experimental subjects. By inspection of the data (Fig. 1) it appears that the acidophilia is not a very precise quantitative criterion for ACTH under the circumstances of the treatment described herein.

Discussion. The results of this study differ in a number of respects from other reports on blood cell changes in birds following injection of ACTH or adrenocortical extracts or hormones. Shapiro and Schechtman(10) reported that injection of adrenocortical extracts into White Leghorn hens resulted in a "leucopenia, absolute and relative lymphopenia and absolute and relative increase in granulocytes attributable mainly to heterophils." Calculation of their printed data also indicates an eosinopenia.

The present results are similar to (although more extensive than) those reported by Wellar and Schechtman(11) who treated embryos with one injection of adrenocortical extract and sampled blood prior to and after hatching (up to 1 month). No lymphopenia was produced, however a marked "polymorphonuclear leukocytosis" occurred 24 hours after injection.

The present results also differ from studies on mammals in which lymphopenia was reported following injection of ACTH in mice, rats, rabbits and human beings(12). Lymphopenia was regarded as a specific response to ACTH since it did not occur in adrenalectomized animals; the polymorphonuclear leucocytosis was regarded as non-specific because it occurred in adrenalectomized as well as intact animals(13).

Details of the physiologic mechanism responsible for the results in this study are unknown. That the leucocytosis was not due to hemoconcentration is indicated by the facts that it occurred in spite of the slightly lowered hematocrit and that the differential counts were markedly changed also.

In spite of the high coefficient of variation and apparent low precision of acidophilia as a *quantitative* criterion of ACTH injection, under the described experimental conditions, results of the two "assay" trials do confirm the use of this criterion as a *qualitative* indicator of ACTH injection and presumably of intrinsic ACTH release.

Summary. Injection of ACTH into 7-14 day old White Leghorn cockerels was followed within 6 hours by a marked absolute leucocytosis and heterophilia and a slight lymphocytosis; no consistent change in numbers of eosinophils, basophils or monocytes was evident within 24 hours. A slight decrease in hematocrit was also produced. The magnitude and constancy of the acidophilia obtained following ACTH injection suggests its possible use as a qualitative criterion of ACTH and/or acute stress in the bird.

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Probable Polypeptidic Nature of Erythropoietin.* (23555)

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Previous work from this laboratory has been concerned with the erythropoietic stimulating effects of plasma extracts from anemic animals and human subjects(1-4) and from hypoxic hypoxic animals(5). The present communication describes certain chemical and physical properties of erythropoietic stimulating factor (EPF) from anemic rabbits. It is hoped that further study of this factor may reveal information relevant to the isolation and purification of the factor.

Materials and methods. Preparation of plasma extracts. New Zealand rabbits were made anemic by daily subcutaneous injections of 1 ml of neutralized (with NaOH to pH 7.0-7.5) (2.5%) aqueous solution of phenylhydrazine hydrochloride until the rabbits' hemoglobin was less than 5 g %. Plasma drawn in heparin (20 mg/100 ml blood) was pooled and frozen at –5°C until approximately 1 l was collected. It was then deproteinized by a modification of the method of Borsook *et al.*(6). The frozen plasma was melted and adjusted to pH 5.5 with 1 N HCl. It was then deproteinized in batches of 250 ml by placing in boiling water for 15 min. The precipitate, which was removed by filtration, was washed with 400 ml of boiling distilled water which was again placed in boiling water for 5 min. before filtration. The filtrates and washings were combined and concentrated *in vacuo* in a flash evaporator to ⅓ the original plasma volume (T < 55°C). Control plasma was obtained from normal untreated rabbits and deproteinized in the same manner. Extracts which received only this treatment are called "pH 5.5 extracts". Some extracts were treated further with trichloroacetic acid (TCA extracts) or by heating at pH 9 (pH 9 extracts). The preparation of the TCA extract was done essentially according to the method of Borsook *et al.*(6). To 1 volume of pH 5.5 extract at 5°C was added 0.1 volume of 50% TCA. After filtration the filtrate was extracted 3 times with equal volumes of ether to remove the TCA. Dissolved ether was removed in the flash evaporator. It was then dialyzed in 36/32" Nojax casing against running tap water for 48 hrs. (T = 9°C). An easier procedure which, however, removed less protein was afforded by heating at pH 9. Alkali (1 N NaOH) was added to the pH 5.5 extract until pH 9 was reached. The solution was then placed in a boiling water bath for 5 min. After the small amount of precipitate had been removed by centrifugation, it was dialyzed against running tap water. *Dialysis.* Dialysis was performed at 5°C against an equal volume of 0.16 M NaCl for 48 hrs. Plasma extract was contained in 20/32" Nojax cellulose casing. Both the dialysate and the dialysand (inside the casing) were tested.

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TABLE I. Effect of Dialysis on Deproteinized Rabbit Plasma Extract (pH 5.5 Extract) Obtained from Anemic Rabbits and Assayed for Erythropoietin Activity in Rats Hypophysectomized at 60 Days of Age.

Fraction administered	No. animals	Avg % 24-hr Fe^{59} uptake and stand. dev.*
Exp. No. 1		
Anemic dialysate	5	35.3 ± 6.56
" dialysand	4	75.1 ± 5.88
" undialyzed	5	82.7 ± 6.48
Saline control	5	30.8 ±11.9
Untreated control	5	35.3 ± 7.64
Exp. No. 2		
Anemic dialysate	5	30.1 ± 4.12
" dialysand	4	72.9 ± 4.66
" undialyzed	5	69.2 ± 3.9
Saline control	5	37.9 ± 8.76
Untreated control	4	33.4 ± 6.65

* Hematocrits and reticulocyte counts were normal for 60-day-old hypophysectomized rats in every case and showed no consistent alteration as a result of EPF administration.

Effect of pH. Separate portions of plasma extract were adjusted to pH's 1, 9 and 13 and placed in boiling water for 5 min. After removal of the precipitate by centrifugation, the pH of the supernatant was returned to 5.5. *Effect of oxidation-reduction.* Mild oxidation was attempted by bubbling oxygen through plasma extract for 1 hr. Similarly, the effect of a mild reducing agent was tested by making the solution 1% with respect to cysteine. *Extractability.* Plasma extract was extracted twice with equal volumes of chloroform, breaking the emulsion formed by centrifugation. Dissolved chloroform was removed from the aqueous fraction in the flash evaporator. Evaporation of the organic layer left a greasy residue showing that this step did remove some material. This was not tested. *Enzymatic digestion.* Separate portions of a dialyzed pH 9 extract were incubated with pepsin at pH 2.0 and with trypsin and chymotrypsin at pH 7.5 Sufficient crystallized enzyme was added to attain a concentration of 0.6-0.8 mg/ml. Incubation was continued for 4 hrs. at 37°C. Controls contained no enzyme. One-half of each enzymatic digest was dialyzed against running tap water for 48-72 hrs. *Bioassay of erythropoietin.* Adult Sprague-Dawley rats aged 2-3 months and ranging in weight from 200-225 g were hypophysectomized. They were maintained on a diet consisting of Purina Laboratory Chow and bread and milk. Either 15 or 60 days after hypophysectomy each rat received 2 cc of plasma extract daily for 3 successive days subcutaneously. Twenty-four hours after the last plasma injection 1 μc of Fe^{59} in 1.0 ml of saline was given intravenously via jugular vein and 24 hours thereafter the rats were bled from the dorsal aorta. Radioactivity in the sample was measured in a Nancy Wood well-type scintillation counter. Radioactivity in an aliquot of the original Fe^{59} solution given each animal was similarly measured. Calculation of Fe^{59} uptake into red cells was carried out as described by Elmlinger *et al.* (7). Precaution was taken after each experiment to run hematocrits. Any animal with low hematocrit was not included in our results since animals with low hematocrits tended to exhibit higher iron uptakes.

Results. The results of the dialysis experiments are shown in Table I. Although employment of 60 day post-hypophysectomized rats caused the control values to be quite high, it is apparent ($P < 0.01$) that EPF did not dialyze through the membrane in detectable quantities.

From Table II it is obvious that EPF is unstable at pH 1 and 13 at 100°C. After this treatment there was little activity left in either the supernatant or the precipitate (data not shown). EPF was much more stable at pH 9 at 100°C, for while there was a statistically significant decrease in activity when compared to that of the untreated pH 5.5 extract, the drop was not pronounced. EPF is not markedly affected by oxidation-reduction or by extraction with chloroform. As can be seen from Table II there was a barely significant decrease in activity after these treatments. A TCA extract was also tested in a similar manner with substantially the same results.

The effect of enzymatic digestion on bioactivity is shown in Fig. 1. Pepsin, trypsin and chymotrypsin caused a pronounced decrease in activity as compared to their controls at the same pH. Upon dialysis of the digestion mixture activity rose in all 3. In

TABLE II. Effect of Various pH's, O_2, Cysteine and Chloroform Extraction on Erythropoietin Activity in pH 5.5 Extract Assayed in Adult Rats 15 Days after Hypophysectomy.

Treatment	No. animals	Avg % 24-hr Fe^{59} uptake and stand. dev.*
Plasma from anemic rabbits		
Supernatant from pH 1, 100°C, 5 min.	6	11.2 ± 7.09
Supernatant from pH 9, 100°C, 5 min.	7	46.1 ± 2.92
Supernatant from pH 13, 100°C, 5 min.	7	10.0 ± 6.42
O_2 bubbled through for 1 hr, 25°C	7	48.6 ± 3.18
1% cysteine added	6	47.6 ± 8.68
Aqueous fraction from chloroform extraction	7	48.8 ± 8.53
pH 5.5 extract, untreated	6	55.7 ± 7.03
Plasma from normal rabbits		
pH 5.5 extract	4	7.4 ± 4.33
Untreated controls	10	2.7 ± 1.73

* Hematocrits and reticulocyte counts were normal for 15-day hypophysectomized rats in every case and showed no consistent alteration as a result of erythropoietin administration.

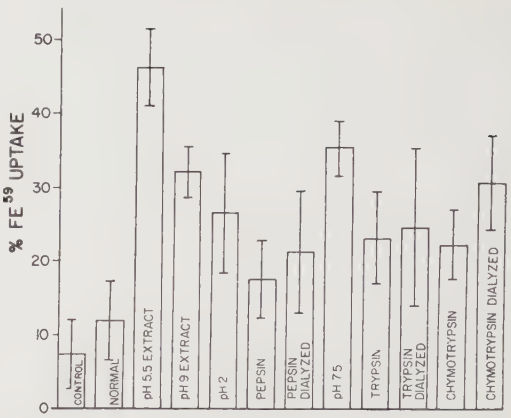

FIG. 1. Erythropoietic activity of various plasma extracts before and after treatment with proteolytic enzymes (see text for details). Averages and stand. dev. of 3 separate experiments are presented.

the case of trypsin the rise was not statistically significant, but in the case of pepsin and chymotrypsin it was ($P < 0.05$ and < 0.01 respectively). Parenthetically, the pH 9 extract again possessed less activity than the pH 5.5 extract.

Discussion. The evidence indicates that EPF is a low molecular weight protein, or polypeptide, perhaps in the range of insulin or ribonuclease. Like ribonuclease(8), corticotrophin(9) and pituitary EPF(10) it is stable to high temperatures in weakly acidic or alkaline solution and like corticotrophin (11) it is stable to and not precipitated by TCA in the cold. However, it is inactivated by heat in strongly acidic or alkaline solution.

Like oxcorticotrophin and ribonuclease, EPF does not dialyze appreciably in water or saline solution at neutral pH. Dialysis against dilute acetic acid which markedly increases the dialyzability of corticotrophin(9), has not been performed yet. Van Dyke *et al.* (10) also found pituitary EPF to be nondialyzable.

It is not certain how stable EPF is to oxidation and reduction. Although the data indicate a fair degree of stability, the presence of large amounts of protein may exert a modifying influence. Levels in plasma expressed as g% protein were: protein, 13; NPN, 30. Levels of total protein in various fractions were as follows: pH 9 extract, 77; TCA, extract, 32; pepsin treated and dialyzed, 25; trypsin treated and dialyzed, 8; chymotrypsin treated and dialyzed, 28. The lack of extractability with chloroform eliminates the possibility of EPF being a lipid.

All of the relationships discussed above are consonant with EPF being either a polysaccharide or a polypeptide. The digestion of EPF by proteolytic enzymes is in accord only with the latter possibility. However, the possibility of EPF being a glycoprotein or a lipoprotein is not excluded. The rise in bioactivity after dialysis of the digestion mixture is interesting. While such observations remain unexplained, conclusions regarding the polypeptidic nature of erythropoietin should be tentative.

Summary. Plasma from anemic rabbits containing erythropoietic stimulating factor was treated in numerous ways in order to determine some of the properties of the factor. Assay was performed by determining the Fe^{59} uptake in hypophysectomized rats. Since the factor remained after heating at pH 5.5 or pH 9 but was destroyed at pH 1 and pH 13 and since it was nondialyzable yet digested by pepsin, trypsin and chymotrypsin, it was

concluded that erythropoietin is probably a polypeptide. In the presence of the non-precipitable proteins (by heat) of plasma, it is fairly stable to mild oxidation and reduction.

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Factors Affecting Development of Hypertensive Vascular Disease After Renal Injury in Rats.* (23556)

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Hypertensive vascular disease may result from various types of renal injury in the experimental animal and often is associated with primary renal disease in the human, but the responsible mechanisms remain unclear. Furthermore, following apparently identical renal injury, incidence and severity of hypertension show considerable variation; *i.e.*, in rats after figure-of-eight tie of one kidney and contralateral nephrectomy, hypertension ranging from mild and chronic form to severe and rapidly fatal disease may develop(1,2). Whether this variability is related directly to extent of renal injury is not readily apparent, since precise studies of its relationship to degree of impairment of excretory renal function, to hypertrophy of remaining renal tissue, or to other factors, have not been reported following this particular injury, and studied in only a limited fashion with other technics for renal injury in rats(3-5). The present study was undertaken to investigate this relationship of renal injury to severity in rats with a figure-of-eight tie of one kidney and contralateral nephrectomy. In addition, the effect of a behavioral disturbance devised to produce a chronic stress for the rat was evaluated.

Materials and methods. Fifty-four male Holtzman rats weighing 150 to 250 g were subjected to "figure-of-eight" tie of the right kidney and, a week later, to a left nephrectomy, according to the technic described by Grollman(1). The animals were lodged in groups of 8 to 10/cage and allowed free access to food and water. Under light ether anesthesia, systolic blood pressure was determined biweekly by tail plethysmographic method(6,7). Renal function was studied prior to sacrifice by testing phenosulfonphthalein (PSP) excretion and urine concentrating ability. PSP excretion was measured by injection of 10 ml of sterile 0.2% saline, intraperitoneally, followed one hour later by a second 10 ml with 1.5 mg of PSP dye; urine was collected over a 3-hour period and percentage of dye excreted determined spectrophotometrically. To determine urine concentration, rats were kept in metabolic cages without food and water for 48-hour period and osmolal concentration of samples col-

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TABLE I. Average Value of Blood Pressure, Renal Function, and Organ Size in 17 Rats with Renal Injury.

	Rt. Fig. 8; Lt. Np.		Intact control		
	Mean	S.D.	Mean	S.D.	p
Systolic B.P. (mm Hg) (avg after onset of hypertension)	146	13.8	110 *	10	.001
Systolic B.P. (mm Hg) (avg of maximum)	160	16.0			
PSP excretion (%)	49.9	13.5	62.9	10.1	.01
48 hr concentration (milliosmoles/l)	1773	611	2413	284	.001
BUN (mg/100 ml)	51.8	14.5	28.7	3.0	.001
Heart wt (mg/100 g)	324	46.2	252	17.8	.001
Kidney " "	619	129.2	648 †	54.3	
Adrenal " "	15.2	2.8	11.1	1.5	.001

* This blood pressure is the avg obtained from several hundred normal animals in this laboratory. Consistent or even sporadic elevations to 140 mm Hg or over, have been rarely observed in our experience nor by other investigators employing this method(8,9).

† Wt of 2 normal kidneys.

lected between 24 and 48 hours measured in a Fiske osmometer. Rats were sacrificed by administration of nembutal and rapid exsanguination by cardiac puncture. Blood urea nitrogen (BUN) was determined on heart blood. The heart, kidney and adrenals were cleaned of extraneous tissue and weighed on a Sartorius balance. The kidney was fixed in 10% formalin, and sections prepared with hematoxylin-eosin, and Mallory stains. The 54 animals were divided into a control group of 24 and a stressed group of 30. Beginning 7 to 10 days after nephrectomy, animals in the stressed group were isolated in cubicles measuring 7 cubic inches for 6 hours 3 times a week. The floor of each cubicle consisted of a brass grid and buzzer was attached to top of each unit of 12 cubicles. Grid and buzzer were activated by a cam-driven automatic timing mechanism. During each 6 hour period in the cubicles, the following pattern was repeated 3 times: For one hour, the buzzer (conditioned stimulus) was sounded at 30 second intervals for 5 seconds, followed, with a 0.5 second overlap, by mild non-convulsive shock (unconditioned stimulus) delivered through the grid. For the next hour, CS was presented alone at 30 second intervals. The weekly procedure was administered for 12 weeks, followed by 6 weeks of rest and then repeated; in animals which survived to end of study, stress was discontinued 3 weeks prior to sacrifice so that renal function tests could be performed. After several trials in the apparatus the animals responded to CS with a "startle" reaction and later in the experiment often appeared to "freeze" in one position. These responses were irregularly extinguished during the period when CS alone was sounded. The control group was kept in colony cages in the same experimental room, and thus experienced the noise of the buzzer, but it produced no apparent response in them.

Results. Thirty-seven animals, including 12 (33%) which failed to become hypertensive, died before completion of the study; their average post-operative life was 20 weeks. The psychological stimulation did not influence mortality rates; of the 37, 21 (70%) were stressed, and 16 (67%) were not. Seventeen animals, 9 under stress and 8 controls, survived with an average post-operative life of 36 weeks. All 17 surviving animals became hypertensive and showed evidence of renal impairment; the average values are summarized in Table I and compared with those from a similar number of normal animals of same age and source as the experimental group. Impaired PSP excretion and decreased osmolar concentration correlated with elevated BUN (Fig. 1). The ligated kidney hypertrophied but failed to compensate for impaired renal excretory function. Actually animals with the most hypertrophy demonstrated greatest impairment of concentrating ability and highest BUN (Fig. 2). Levels of blood pressure did not demonstrate a linear correlation with any of the renal function measurements or with renal mass, with the exception of a sug-

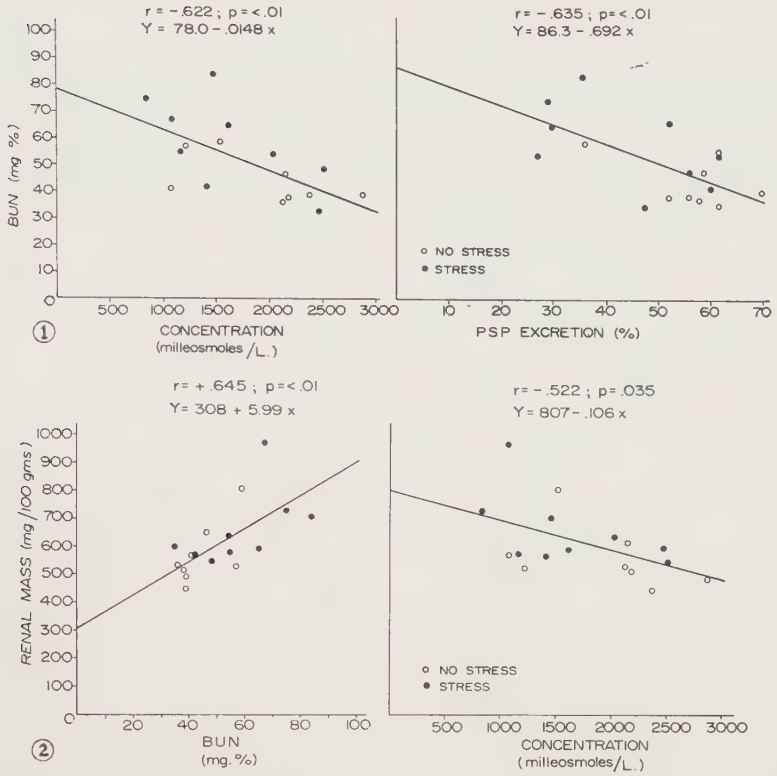

FIG. 1. Graphs indicate the negative correlations between BUN and concentrating ability, and BUN and PSP excretion. Each point represents one rat; stressed and non-stressed animals have been combined to form one group for these comparisons, but are charted with different symbols.

FIG. 2. Graphs indicate positive correlation between wt of the kidney (renal mass) and BUN, and negative correlation between renal mass and concentration. Each point represents one rat.

gestively positive relationship between maximum blood pressure and renal weight ($r = +.482$; $p = .05$). Although levels of blood pressure in both stressed and nonstressed animals did not differ, the severity of hypertensive disease as evidenced by heart size, renal excretory function and adrenal size, seemed more pronounced in the former (Table II). The onset of hypertension, as calculated from the number of weeks after surgery when a systolic blood pressure of 140 mm Hg or over was first noted, averaged 14.1 wks. in the stressed and 25.5 wks. in the nonstressed group. A 4-fold test of contingency(10), revealed a difference significant at the 5% level between number of animals in each group who were hypertensive at the 20th week. Duration of hypertension did not account for differences between stressed and

TABLE II. Comparison of Onset, Duration, and Severity of Hypertension in Operated Rats with and without "Psychological Stress."

No. of animals→	Stressed 9		Nonstressed 8	
	Mean	S.D.	Mean	S.D.
Avg age at sacrifice (wk)	47.7	13.8	47.3	13.1
Avg post-operative life (wk)	36.8	11.8	35.8	9.9
Avg systolic B. P. (mm Hg)	142	16.9	150	8.6
Avg max systolic B.P. (mm Hg)	159	19.0	161	12.9
Heart wt (mg/100 g)	349	38.7	296	37.5 *
Kidney wt "	663	133	570	113
Adrenal wt "	17.0	2.58	13.3	2.14*
BUN (mg/100 ml)	58.3	15.8	44.4	8.9 †
48 hr conc. (milli-osmoles/l)	1614	658	1941	601
PSP excretion (%)	44.1	14.1	56.5	9.70†

* Significant differences at 1% level.
† Probably significant differences at 5% level.

nonstressed groups, since it did not demonstrate a correlation with measurements of organ weight, renal function, or blood pressure ($p > .05$ in all instances).

Pathological changes. All components of the kidney nephron were affected, but the damage was most consistent in the tubules. Flattening of the epithelium in the proximal tubules, dilatation of distal and proximal elements, and accumulation of eosinophilic material in their lumens were noted. Scattered infiltrates of mononuclear cells were present throughout the kidney, but never to the degree nor with the scarring seen in experimental pyelonephritis in the rat(11). The glomeruli were well preserved and demonstrated only periglomerular fibrosis and hypertrophy until overall damage was moderate to severe, and then varying degrees of fibrosis ranging to complete replacement were noted. In the mildly affected kidneys, arteriolar damage was absent, but medial thickening and hyalinization were present in the moderate to severe group while in the worst kidneys intimal thickening, and occasionally typical "onion peel" lesions of nephrosclerosis, were apparent. Classification of the severity of damage to each kidney on the basis of 1+ to 3+ revealed the following:

	1+	2+	3+
Stress group*	0	4	4
Nonstress group	5	2	1

* Tissue was available for study in only 8 of the 9 animals in the stress group.

Discussion. Presence of excretory renal impairment and yet its failure to correlate directly with severity of hypertension is not a new or unexpected finding although the quantitative aspects of the relationship have not been previously demonstrated in rats with a figure-of-eight tie of one kidney and contralateral nephrectomy. The hypertrophy of the constricted kidney was striking, and it is of interest that it occurred despite injury to the kidney and that it failed to compensate for functional impairment. Nor did renal hypertrophy prevent the development of hypertension, indicating that if an increatory function of the kidney regulates blood pressure(12), it is not a function of renal mass. Since excretory renal impairment alone does not correlate directly with the severity of the hypertension, other factors must interact to determine the blood pressure regulatory function of the kidney and to influence the course of the disease. The seemingly adverse effects of the noxious psychological stimuli suggest one such factor. Ample clinical evidence indicates that psychological turmoil can play a role in the precipitation of acute changes in blood pressure and the progression of hypertensive vascular disease in man(13,14). The present experiment offers evidence in the experimental animal of these inferences.

Summary. Correlations between renal excretory function (PSP excretion, osmolal concentration of urine, and BUN), renal mass and hypertension were studied in 17 rats which had been subjected to a figure-of-eight tie of one kidney and contralateral nephrectomy. In addition, 9 of the animals were exposed to the stress of a psychological disturbance. All 17 animals became hypertensive and all had some impairment of excretory renal function. However, the degree of impairment of renal function did not correlate with the level of blood pressure. Hypertrophy of remaining renal tissue was marked but did not prevent functional impairment or blood pressure elevation. The noxious psychological stimulus appeared to worsen the manifestations of the hypertensive process.

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Effect of Physical Activity on Cholesterol Atherosclerosis in Rabbits.* (23557)

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Cholesterol atherosclerosis has been produced in animals by many investigators. The results of these experiments and of studies in the pathogenesis of the lesions have been extensively reviewed(1-4). In man the effect of diet on serum lipids has been extensively investigated. However, opinion varies on the importance of all lipids(5) and of the type of ingested lipid(17-21) causing variations of serum lipids in man. The serum lipid levels and the serum lipoprotein types have some relation to the occurrence of sequelae of atherosclerosis, but the predictive value and role of these factors in the pathogenesis of human atherosclerosis are not defined(7). In human beings, as well as in experimental animals, very little attention has been paid to the question of the influence of physical activity of the atherosclerotic process. The effect of physical activity on incidence of coronary artery disease in sedentary and more active workers was studied, and although the groups differed in respects other than those of physical activity, it was judged that the active workers had less severe heart disease than the more sedentary workers(6). Some indication was obtained that, following ingestion of a high fat meal, exercise lessened the degree of hypercholesterolemia(9). In studies of different national groups, a tendency to a lowered serum cholesterol was found in Bantus doing heavy manual labor, but in white populations, total fat content had more effect on cholesterol level in the blood than did exercise(8). In experimental animals there is, to our knowledge, only one study on record concerning the effect of physical activity on rabbits being fed cholesterol(10), in which the sedentary and exercised groups showed equal atherosclerosis. In our experiments an apparent decrease in the amount of atherosclerosis of the aorta of cholesterol-fed rabbits was shown to occur in the group of rabbits exercised in an electrified treadmill.

Materials and methods. The animals used in this experiment were New Zealand white rabbits purchased from a local supplier.‡ They consisted of 5 groups of 5 to 7 litter mates obtained from bucks and does of known pedigree. The animals were individually housed in air-conditioned animal quarters. Each litter of 5 to 6 animals was divided into 2 groups without reference to sex. One was fed cholesterol and allowed to remain sedentary. The other group was fed cholesterol but exercised. The sedentary and experimental groups are indicated in Table I and sex distribution is evident in Figs. 1-6. The inclusion of a group of animals fed an ordinary diet was not considered necessary since this strain of rabbits has been used for years in this laboratory for various purposes.

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‡ Grables Rabbit Ranch, Pontiac, Mich.

TABLE 1. Visual Grading and Lipid Content of Aortas.

	Sedentary			Exercised			
Animal #	A 9	A 10	A 11	A 6	A 7	A 8	
Visual grade	++++	++++	++++	+	+	+	
Total cholesterol, mg %	30.3	26.9	28.0	9.0	5.1	7.4	
Free " "	11.4	12.9	6.3	4.6	3.2	3.9	
Lipid phosphorus, "	.39	.48	.42	.22	.22	.19	
Aorta wt, g	1.11	.96	.94	.87	.67	.73	
Animal #	B 7	B 9	B 11	B 6	B 8	B 10	
Visual grade	+++	++	++	+	+	+	
Total cholesterol, mg %	12.7	4.3	7.0	11.1	3.8	5.7	
Free " "	4.0	1.9	3.0	5.7	1.8	2.8	
Lipid phosphorus, "	.24	.15	.18	.33	.16	.19	
Aorta wt, g	.80	.58	.50	.96	.63	.70	
Animal #	E 2	E 4	E 6	E 1	E 3	E 5	
Visual grade	+++	+++	++	+	++	++	
Total cholesterol, mg %	22.3	19.4	13.6	8.3	14.3	15.6	
Free " "	7.2	5.7	4.1	3.4	5.0	4.3	
Lipid phosphorus, "	.16	.03	.17	.18	.10	.20	
Aorta wt, g	.82	.75	.66	.60	.71	.78	
Animal #	G 3	G 5	G 6	G 1	G 2	G 4	G 7
Visual grade	++	++	++	+	+	++	+
Total cholesterol, mg %	10.4	6.9	7.3	8.9	2.6	20.6	1.8
Free " "	5.7	2.0	2.3	2.5	1.6	5.9	.6
Lipid phosphorus, "	.14	.15	.16	.19	.15	.23	.12
Aorta wt, g	.53	.52	.60	.67	.58	.87	.54
Animal #	H 1	H 4	H 5	H 2	H 3	H 6	
Visual grade	+	+	++++	+	+	++	
Total cholesterol, mg %	1.0	1.6	24.9	4.4	5.0	15.2	
Free " "	.6	.7	9.2	1.8	2.7	5.4	
Lipid phosphorus, "	.03	.07	.41	.09	.14	.27	
Aorta wt, g	.49	.48	.90	.59	.67	.71	
Animal #	J 2	J 3	J 4	J 1	J 5		
Visual grade	+++	—	++++	+	+		
Total cholesterol, mg %	11.0	1.3	17.8	5.3	2.7		
Free " "	3.8	.5	4.3	2.9	1.2		
Lipid phosphorus, "	.21	.07	.29	.07	.14		
Aorta wt, g	.63	.42	.69	.53	.61		

has been fed the same diet free of cholesterol and has shown no tendency to develop atherosclerosis of the aorta. The rabbits were fed cholesterol for ninety days: 75 g of ordinary Purina Chow in the evening and 25 g of rabbit chow coated with 0.25 g cholesterol in the morning. The pellets were prepared by mixing them with an appropriate solution of cholesterol in ether and allowing the ether to evaporate, leaving the pellets coated with the cholesterol in the amount of 1 g of cholesterol per 100 g of pellets. Any residual food from either feeding was to be weighed. However, throughout the experiment this did not prove to be necessary since all the rabbits invariably ate their full ration. Water was given *ad libitum*. Total amount of cholesterol fed throughout the experiment was 24.25 g of cholesterol, 0.5 g per day for 7 days and 0.25 g per day for 83 days. *Blood Chemistry*. All animals were bled every two weeks when 10 cc of blood was collected from the central artery of the ear before feeding or exercise on the day of bleeding. The methods used were: for cholesterol determination, that of Zak *et al.*(13); for total lipids, Kunkel *et al.*(14); and for lipid phosphorus, modifications of the procedures of Youngburg and Youngburg(15) and Fiske and Subbarow(16). *Exercise*. An electrified drum¶ was constructed, 30 inches in diameter and 9 inches wide, in which the peripheral wall of the drum was composed of 3 16 inch brass

¶ Constructed by Ernest Artt, Detroit, Mich.

FIG. 1-4.

FIG. 1-6. Aortas from the animals by litters. The exercised animals are marked with an asterisk. Sex and visual grading are indicated in the diagrams. Note the tendency to diminished atherosclerosis in all aortas marked with an asterisk.

rods, placed 5/8 of an inch apart. These were wired alternately and connected to a power source (Electro-stimulator§) that delivered a damped-wave alternating current at 2,000 to 4,000 volts and 1 to 2 milliamperes to the rabbits. It was found necessary to shave or clip the feet of the rabbits closely so that

§ Constructed by M. L. Appelbaum, Detroit, Mich.

FIG. 5 and 6.

shocking might be possible since rabbits do not have pads on their paws (a fact apparently little known). Electrode jelly, such as used for electrocardiograms or electroencephalograms, proved ineffective as a conductor for electrical impulses to the paws. By shocking the animal and, at the same time, sounding a bicycle horn, the rabbits could be conditioned to run at the sound of the horn. In this manner they could be induced to rotate the drum about 50 times per minute. The animals were exercised in this way for 5 minutes twice daily before food was given. The animals were obtained 4 weeks before the exercise was begun and all were exercised 4 weeks, until 2 weeks prior to the beginning of the cholesterol feeding when the exercise was stopped in the sedentary group. *Autopsy.* After 13 weeks a complete autopsy was performed. The aortas were fixed in formalin and the adventitia meticulously cleaned. The extent of the atherosclerosis was drawn without preliminary staining in Sharlach solution as it was not desired to lose any of the lipid in the stain solvent. The aortas were then subjected to ether alcohol extraction according to Gordon *et al.*(11) and the cholesterol and phospholipids determined as for serum. *Visual grading* of the aortas was done according to standards outlined by McMillan *et al.*(12).

Results. Degree of atherosclerosis of the aorta. Inspection of Figs. 1-6 indicates that, estimated visually, no animal of any exercise group ever developed atherosclerosis of more than 2+, whereas half the animals of the sedentary group developed atherosclerosis of 3 and 4+.

The *lipid content of the aortas* is indicated in Table I. The correspondence between content of lipids and visual grading is by no means absolute, but the tendency for higher values in the sedentary group is fairly consistent. In the J group where the greatest inconsistency is present in the degree of sclerosis, the animals were not litter mates nor of the same breed. That is, they were not all New Zealand whites, but mixed Dutch, checkered giant and mongrel. It is also interesting that there was no correlation be-

TABLE II. Gain and Loss of Body Weight and Adipose Tissue in Retroperitoneum and Omentum.

	Sedentary				Exercised			
	Body wt (g)			Adipose	Body wt (g)			Adipose
	Start	End	Gain	tissue	Start	End	Gain	tissue
Mean	2325	2791	466	++ - +++	2492	2863	371	0 - +*
Range	1965–2840	2325–3300	245–740		2235–2872	2520–3160	145–735	

* One animal only.

tween the *sex* and the degree of atherosclerosis. Some of the females in a given litter developed more atherosclerosis than the males (Fig. 1-6). It is considered an important observation that *in the exercised rabbits* there was a significant *diminution in the amount of fat in the depots* in contrast to those of the sedentary animals. The muscles of the exercised rabbits were also more hyperemic than those of the sedentary group. Although the exercised group was leaner, there was no significant difference between the weights of the animals in the two groups (Table II).

Serum lipids, the total cholesterol, phospholipids, cholesterol esters, as well as the total cholesterol to phospholipid ratios were determined and calculated for each animal. There was no correlation between any of the lipid levels in the blood and the degree of atherosclerosis in the aorta, in individual animals or when the average results of all the animals in the sedentary or exercised groups were plotted.

Discussion. In a previous experiment several methods of exercise were tried.‖ The first was an apparatus decribed by Kramar (*22*), whereby a large drum was rotated slowly by means of an electric motor. We were unable to induce rabbits to run normally in such an apparatus. Even if it was revolved slowly at 4 or 5 revolutions per minute, they soon began to slide and tumble on the rubber matting. They became almost paralyzed with fear, and once exposed to such an experience, the rabbits would only tumble passively in the apparatus. When the motor was detached and the drum rotated manually, it was an exceptional accomplishment to even induce a rabbit to walk in the drum. Lightweight drums designed to be rotated by the rabbit were equally ineffective. Despite extreme caution, mechanical treadmills imposed a stressful experience for animals that could not be properly considered exercise. No exercise effect was observed either on the body or on the deposition of atherosclerosis.‖ The efficacy of the exercise in the present experiment is attested to by the leanness of the rabbits at autopsy on the standard food intake and the general hyperemia of their muscles. It may be that the development of this apparatus is a significant contribution of the experiment. The difference in the type of exercise thus possible may account for the difference between our results and those of Brown *et al.*(10).

It should also be noted that after the first week of the experiment the amount of cholesterol given to the rabbits was only 0.25 g per day. This was done on the theory that whatever mechanisms allowed the deposition of cholesterol in the aorta should not be overwhelmed by too large a dose of the cholesterol, so that any possible effect of exercise in prevention might become apparent.

The amount of atherosclerosis present in the exercise group appears to be distinctly less than in the sedentary group. The use of the lipid content of the entire aorta to correlate with the visual grading follows the recommendations of McMillan, Horlick and Duff(12) who discussed the controversial considerations fully. They concluded that both visual and chemical estimations of the degree of atherosclerosis should be used to evaluate these experiments. Brown *et al.*(10) used evaluation of microscopic sections of aorta, likely to be subject to sampling errors.

These results of our experiment are not absolute in that atherosclerosis developed in the exercise group. However, if it is indeed true that exercise is a significant factor in

‖ Unpublished data.

the reduction of the amount of atheromatous deposit in the aorta, then it is also probable that it is not the only factor in the pathogenesis of atherosclerosis in rabbits.

It is interesting that the effect of physical activity was more evident in the relatively pure-bred litters (litters A, B, E, G, H) than in the unrelated animals of group J. This implies the possibility that some individual strain factor exists in the amount of atheromatous deposition, which is also not unexpected. This may also account for the disparity of these results and those of Brown *et al.*(10) in which animals were used at random. The absence of correlation of the atheromatous deposits with the level of the serum lipid is consistent with what may be expected in view of the extreme controversy in which such a correlation is held both in human beings and in experimental animals(3).

The results of the present experiment are considered to be highly suggestive of an effective diminution of the deposit of atheroma in the aorta of cholesterol-fed rabbits but further confirmatory experiments are required to confirm and to establish the pathogenesis of this phenomenon.

Summary. 1. Thirty-six New Zealand white rabbits of 5 litters and one group of miscellaneous rabbits were fed 28 g of cholesterol for 60 days. Half of the animals were exercised for 10 minutes a day; half the animals remained sedentary. 2. There was a significant diminution of atherosclerosis in the exercised animals as determined by visual grading and by chemical analysis of the lipid content of the aorta.

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Plasma Ultrafiltrable Magnesium in Respiratory Alkalosis and Acidosis.* (23558)

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Recently changes in the plasma ultrafiltrable calcium during and following hyperventilation and respiratory acidosis have been reported(1,2). A rebound increase in level of ultrafiltrable calcium in the recovery phase following hyperventilation and a similar but inverse rebound following respiratory acidosis experimentally produced in dogs were observed. It was also considered desirable to investigate the status of ultrafiltrable magnesium under these conditions.

Methods. Nine mongrel dogs anesthetized with thiopental sodium were used in this study. Five were subjected to 30 minutes of hyperventilation produced by a positive pressure respirator set to deliver 20 cm of water pressure at a rate of 30-40 breaths/minute and 4 were made acidotic by breathing 30% CO_2 and 70% O_2 from an open system for 30 minutes. Forty ml blood samples were drawn under oil from the femoral artery into a tube containing powdered heparin. Plasma was separated under oil. Samples were drawn before, at the end of the 30 minute experimental period, and 25 minutes after return to normal breathing in both sets of experiments. The method of obtaining an ultrafiltrate of plasma with a minimum shift in pH during ultrafiltration has been previously described (3). Magnesium in plasma and ultrafiltrate was determined(4). Phosphate was determined by the method of Fiske and Subbarow (4). Plasma pH was determined by means of anaerobic electrodes using a research model Cambridge pH meter at 37°C. In 4 *in vitro* experiments 160 ml of freshly drawn heparinized dog blood was divided into four 40 ml aliquots. These portions of blood were placed in 500 ml tonometers and equilibrated with various gas mixtures as follows: Sample I, 5% CO_2—95% O_2; II, 30% CO_2—70% O_2; III, 30% CO_2—70% O_2 followed by 5% CO_2—95% O_2; IV, 5% CO_2—95% O_2. Monobasic sodium phosphate was added to each of the latter 3 samples in amount sufficient to raise plasma concentration to approximately 14 mg%. Equilibrations with gas mixtures were carried out in a water bath maintained at 38°C and were continued for 30 minutes. After 15 minutes of equilibration the proper gas mixture was flushed through the tonometer a second time. In the case of sample III, equilibration with 30% CO_2 mixture was carried out for 30 minutes followed by equilibration with the second mixture for 30 minutes.

Results. Table I presents the means, with their standard deviations, for total and ultrafiltrable plasma magnesium concentration and plasma pH range in which ultrafiltration was carried out, for both sets of experiments. Ultrafiltrable magnesium showed a significant increase within 25 minutes after cessation of hyperventilation as compared with the samples obtained prior to and during hyperventilation. Total magnesium showed no significant changes. Total and ultrafiltrable magnesium showed essentially no change during respiratory acidosis but both decreased somewhat following return to air breathing. The *in vitro* experiments (Table II) revealed no significant changes in level of total and ultrafiltrable magnesium in all 4 samples.

Discussion. The changes observed in ultrafiltrable magnesium are similar to that of calcium in hyperventilation experiments. A rebound increase in level of ultrafiltrable magnesium 25 minutes after return to air following hyperventilation was noted. It was suggested(1) that the rebound increase in level of ultrafiltrable calcium following hyperventilation was related to a decrease in serum phosphate and change from alkalosis to normal pH. Recently, an increase in level of

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TABLE I. Mean Values for Plasma Total and Ultrafiltrable Magnesium Concentration in meq/l in 5 Dogs in Gr. I and 4 Dogs in Gr. II. Mean value for pH of the plasma immediately before and after ultrafiltration is tabulated with each value for ultrafiltrable magnesium. P = .05 when mean Uf Mg during hyperventilation is compared with mean Uf Mg 25 min. after hyperventilation. P = .05 when mean Uf Mg during respiratory acidosis is compared with mean Uf Mg 25 min. on air.

I	Initial sample		During hyperventilation		25 min. after hyperventilation	
	Total Mg	Uf Mg	Total Mg	Uf Mg	Total Mg	Uf Mg
	2.18 ± .3	1.39 ± .26	1.95 ± .26	1.21 ± .21	2.07 ± .22	1.60 ± .36
	pH 7.29 ± .06 – 7.54 ± .15		pH 7.52 ± .06 – 7.81 ± .07		pH 7.27 ± .06 – 7.47 ± .06	

II	Initial sample		Breathing 30% CO_2		25 min. on air	
	Total Mg	Uf Mg	Total Mg	Uf Mg	Total Mg	Uf Mg
	1.96 ± .17	1.37 ± .06	2.07 ± .24	1.39 ± .03	1.84 ± .2	1.16 ± .24
	pH 7.37 ± .04 – 7.63 ± .03		pH 6.92 ± .02 – 7.33 ± .06		pH 7.41 ± .06 – 7.62 ± .03	

plasma citrate has been observed following hyperventilation(6). It seems probable that increased citrate level is responsible for the increase in level of ultrafiltrable calcium and magnesium by forming a filtrable citrate complex. Plasma proteins do not change significantly to account for these changes(1).

During acidosis ultrafiltrable magnesium showed no changes. However, total and ultrafiltrable magnesium both showed a slight decrease, 25 minutes on return to air. Thus, changes in magnesium in these experiments were unlike those observed with respect to calcium(1). The ultrafiltrable calcium increases slightly during acidosis and falls below control level following return to air breathing. The total calcium shows no change. The decrease in level of ultrafiltrable calcium during recovery phase is believed to be due to an increase in phosphate level. The *in vitro* experiments (Table II) show that increased phosphate level and pH changes have no effect on ultrafiltrable magnesium. It may be possible that the decrease in total and ultrafiltrable magnesium observed in post hypercapnia period is due to a shift of this ion into the cells.

Summary. (1) Ultrafiltrable magnesium shows a significant increase within 25 minutes after cessation of hyperventilation in dogs as compared to the sample taken during hyperventilation, whereas total magnesium showed no changes. (2) Total and ultrafiltrable magnesium showed no significant changes during respiratory acidosis, as compared to the initial sample. A significant decrease in level of ultrafiltrable magnesium after 25 minutes on return to air breathing, as compared to the sample taken during 30% CO_2 breathing, was noted. A shift of magnesium ion intracellularly is a possible explanation. (3) The results obtained in *in vitro* experiments show that increased phosphate level and pH changes, under the conditions of the experiments, show no effect on ultrafiltrable magnesium level, in contrast to changes previously observed with respect to ultrafiltrable calcium.

TABLE II. *In Vitro* Experiments. Mean values for 5 experiments on dog blood. Stand. error is given with each mean. Samples I and IV were equilibrated with 5% CO_2, Sample II with 30% CO_2 and Sample III with 30% CO_2 and then with 5% CO_2. NaH_2PO_4 was added to Samples II, III and IV before equilibration. Gas mixtures were CO_2 and O_2. Equilibration was carried out for 30 min. at 38°C.

	Total Mg	Uf Mg	Po_4	pH
I	1.78 ± .28	1.50 ± .17	4.7 ± .30	7.28 ± .027
II	1.88 ± .34	1.60 ± .22	14.6 ± .34	6.77 ± .017
III	1.89 ± .40	1.56 ± .34	14.4 ± .31	7.22 ± .019
IV	1.83 ± .38	1.59 ± .28	14.8 ± .40	7.24 ± .018

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Effect of Lysine Provided by Different Routes on Cariogenicity of Lysine Deficient Diet. (23559)

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Evidence of a cariogenic role of heat-processed skim milk powder in diets of white rats has been presented(1-5). In lysine deficient diets a dietary supplement of L-lysine resulted in a reduction in caries(6,7). An unanswered question pertaining to the caries inhibitory effect of lysine under these conditions is its mode of action, *i.e.*, is lysine acting within the oral cavity or does it have a systemic effect which in turn influences susceptibility to dental caries. To obtain information relative to this question, the current experiments have compared the effect of lysine ingested (a) in food, (b) in drinking water, (c) by intubation, and (d) by intraperitoneal injection.

Methods. The general plan of these studies as well as the preparation of the lysine-deficient basal diet 636 are described elsewhere(1,2). Holtzman rats from the National Institutes of Health colony, at weanling age were distributed as litter mates between control and test groups, and continued on experiment sixty days. Teeth were examined under low power (10X magnification) as previously described(4) and only that caries scored, which was observed on buccal and lingual surfaces. The essential data and plan of the experiments appear in Table I. In Experiments 1-a, –b, –c, –d and –e, 5 littermates from 40 litters were housed 2/cage. A regimen of approximately equalized diet intake of paired control and test littermate rats was followed by giving equal quantities of diet to the 2 rats/per cage. Under this regimen, followed also in experiments 2–a and –b, 3–a and –b, rats which had no supplemental lysine consumed *ad libitum* a quantity of diet which established the level of food intake for their littermates receiving supplemental lysine. Rats in both 1–a and –b served as controls, the latter group receiving 1 ml distilled water by intubation 5 days per week. One ml of a solution containing 75 mg L-lysine* was intubated in Exp. 1–c, 5 days/week. The diet and drinking water of rats in group 1–d and –c contained 1.5% of L-lysine. In Exp. 2–b (2–a served as control) one ml of L-lysine solution (75 mg/cc) was intubated 6 days/week. Food was withheld from 5 P.M. to 9 A.M. Two hours after feeding at 9 A.M., the rats were intubated. With this regimen it seemed probable that the intubated L-lysine would be available when food was present in the digestive tract. In experiments 3–b and 4–b, the rats were injected intraperitoneally twice daily 5 days/week (once on sixth day) with one ml of a solution containing 100 mg L-lysine. Rats in 3–a and 4–a were injected with 1 ml of distilled water. In experiments 4–a and –b the rats were housed individually and daily food intakes approximately equalized, for each pair of control and test rats. Food consumption was *ad libitum* in Exp. 5-a, -b, -c, and –d. One ml of a solution containing 100 mg of L-lysine was intubated or injected twice daily 5 days/week (once on sixth day) in Exp. 5–c and –d respectively. The diet in 5–b contained 1.5% of L-lysine. A littermate group of control rats was compared with groups of test rats, each group containing 40 rats and housed 2/cage. All the rats in all experiments were weighed weekly. The growth data for this last experiment are shown in Fig. 1. The approximate daily intake of supplemental L-lysine was as follows: 1–c (intubation) 55 mg, 1–d (diet) 75-80 mg, 1–e (drinking water) 150-180 mg, 2–b (intubation) 65 mg, 3–b, 4–b, 5–c and 5–d (injection or intubation) 160 mg, 5–b (diet) 150-180 mg. It may be noted that in one previous study on the effect of L-lysine added to the diet, 0.25%, 0.50% and 2.50% L-lysine were about equally effective

* As the monohydrochloride.

TABLE I. Effect of Lysine on Cariogenic Property of Diet 636 Containing Autoclaved Skim Milk Powder.

Exp.	Diet regimen and supplement	No. of rats	Avg daily gain* (g)	Caries experience: Rats with caries (%)	Caries experience: Carious lower teeth/rat	Caries experience: Carious areas/rat	Caries experience: Caries score/rat
1 a	Control	38	.55	94.7	4.6 ± .4†	9.1 ±1.0	10.6 ±1.5
b	Water by intubation	37	.57	100.0	5.3 ± .6†	10.7 ±1.4	13.7 ±1.9
c	L-lysine by intubation	29	.88	72.4	2.0 ± .4†	3.2 ± .7	3.4 ± .8
d	" : 1.5% in diet	34	1.00	32.4	.9 ± .3†	1.4 ± .5	1.5 ± .5
e	" : 1.5% in drinking water	33	1.00	45.5	1.1 ± .3†	1.8 ± .5	1.9 ± .5
2 a	Control	30	.75	84.6	2.9 ± .3	5.5 ± .7	6.3 ± .9
b	L-lysine by intubation	39	1.12	38.5	.5 ± .2	1.1 ± .3	1.2 ± .3
3 a	Control	39	.52	87.2	2.4 ± .4	3.6 ± .7	4.0 ± .8
b	L-lysine by intraper. inj.	36	.79	66.7	1.9 ± .3	2.9 ± .6	3.4 ± .7
4 a	Control	40	1.15	47.5	1.0 ± .2	1.3 ± .3	1.4 ± .3
b	L-lysine by intraper. inj.	39	1.35	48.7	1.4 ± .3	2.2 ± .2	2.4 ± .7
5 a	Control	38	.72	100.0	4.9 ± .3	11.0 ± .9	18.7 ±2.0
b	L-lysine: 1.5% in diet	37	2.12	81.1	2.6 ± .3	4.8 ± .6	5.4 ± .8
c	" by intubation	39	1.70	94.9	3.7 ± .3	7.1 ± .7	10.8 ±1.4
d	" by intraper. inj.	38	1.69	100.0	4.7 ± .2	9.4 ± .7	13.0 ±1.3

* Food intake was approximately equalized in all experiments except 5 a, 5 b, 5 c and 5 d.
† Both upper and lower teeth were diagnosed.

in reducing the caries which develops on diet 636(6). The estimated lysine intake in these later studies(6) was 40-200 mg daily.

Results. As was to be expected(6), 1.5% L-lysine in the diet reduced caries significantly (experiments 1–d and 5–b). L-lysine also significantly reduced both incidence and severity of caries when given in the drinking water (experiment 1–e). In three trials, 1–c, 2–b and 5–c intubation of L-lysine also significantly reduced caries severity. The difference in the control score 18.7 (experiment 5–a) and a score of 10.8 (experiment 5–c) is statistically significant, $p < 0.01$. Severity scores were reduced by stomach-tubed L-lysine from 13.7 (1–b) to 3.4 (1–c) and from 6.3 (2–a) to 1.2 (2–b). Incidence of caries was also reduced by lysine intubation from 100.0% (1–b) to 72.4% (1–c) and from 84.6% (2–a) to 38.5% (2–b).

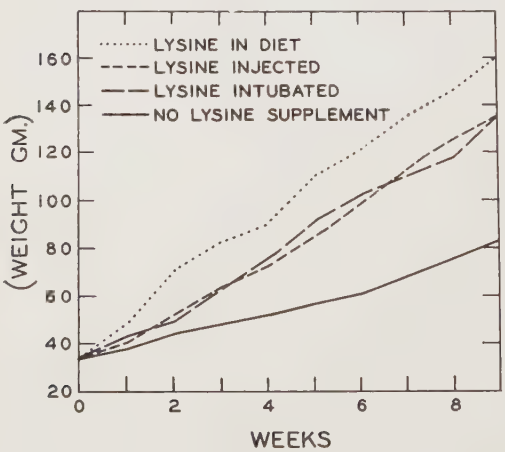

FIG. 1. Growth of rats receiving lysine by different regimens as a supplement to diet 636.

The results of administering L-lysine by intraperitoneal injection were variable, a caries reduction having occurred only in experiment 5–a *vs* 5–d and this was evidenced only in severity, $p < 0.02$. No change in caries was noted in experiments 4–a and –b. In connection with the low incidence of very mild caries observed in experiment 4–a, it may be noted that there is considerable variability in the caries in control groups of rats. This may be explained by differences in rats and by variations in the lysine content of the autoclaved skim milk powder. Different batches of skim milk powder were used and very slight changes in autoclaving conditions, moisture particularly, influence the lysine inactivation. It is generally true, also, that housing one rat/cage, and providing fresh diet daily (as required by control feeding) usually results in a low incidence of very mild caries. Food consumption which is increased by giving this diet fresh daily, as well as housing individually, would ap-

pear to account for the increased rate of growth in 4–a.

Discussion. Lysine in the diet and drinking water was about equally effective in inhibiting caries, followed by lysine administered by intubation. Under the conditions of these experiments, no consistent significant inhibition of caries resulted from injected lysine. Of special interest, however, is the finding of a significant caries-inhibitory effect of intubated lysine. It would seem that a systemic association could be the determining factor in this cariostatic action of lysine, in contrast to a direct effect of lysine localized within the oral cavity.

The possibility that an increase of lysine in rats' saliva could explain the effect of intubated lysine was considered and the following experiment was conducted to help resolve this question.† After injecting 1.0 ml of a solution containing 100 mg lysine intraperitoneally, saliva was collected according to the procedure of Benarde *et al.*(8) with slight modifications. The pooled salivas of 16 rats (about 1 cc per rat in 30 minutes) were analyzed for lysine using ion exchange chromatography on Dowex 50(9). The amount of lysine determined was less than 1.0 mg %, a quantity which seems insufficient to have brought about this significant effect of intubated lysine on the rats' caries experience.

The growth data in Fig. 1 show that under this regimen of *ad libitum* feeding dietary lysine gave the best growth improvement whereas intubated and injected lysine afforded about equal improvement in rate of growth. It is of interest that whereas injected lysine under the conditions of this experiment resulted in no consistent caries reduction, it was utilized for growth (Fig. 1). The result is in keeping with a previous suggestion(7) that the requirement of this amino acid for optimum growth may not coincide with other systemic requirements.

Finally in resolving the complex etiology of dental caries, it may be noted that there is need to extend our information regarding the role of the systemic metabolism beyond the immediate confines of the oral cavity. It is of particular interest, therefore, that a reduction in caries resulted from intubated L-lysine. In this connection it may be mentioned also that in preliminary studies from this laboratory, a caries potentiating effect of intubated EDTA (ethylene diamine tetra acetic acid) has been observed‡ and also according to other experiments,§ DHAS (sodium dehydroacetate) administered by stomach tube as well as by intraperitoneal injection, caused a significant increase in this particular experimental smooth surface rat caries.

Conclusions. (1) Administration of L-lysine in food, water and by intubation, to rats receiving a highly cariogenic lysine-deficient diet containing autoclaved skim milk powder, significantly inhibited smooth surface caries as diagnosed in these experiments. Although L-lysine as administered by injection was utilized for growth, it did not clearly inhibit caries. (2) When administered by intubation L-lysine was cariostatic. The result suggests a systemic involvement in this action of lysine on dental caries. This hypothesis gains support in the fact that injection of lysine resulted in less than 1 mg % lysine in rats' saliva.

† We are indebted to I. Zipkin and H. L. Wolff for this saliva study.

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§ Zipkin, I. and McClure, F. J., unpublished data.

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Electrocardiographic Patterns Evoked by Venom of the Stingray.*† (23560)

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Parenteral administration of stingray venom produces profound changes in the mammalian cardiovascular system. Low concentrations of the venom give rise to simple peripheral vasodilatation or vasoconstriction. High concentrations cause marked vasoconstriction of the large arteries and veins as well as of the arterioles. Much more serious is the direct effect on the heart muscle producing changes in the rate and amplitude of systole. The venom may cause complete, often irreversible, cardiac standstill. It is apparent that the venom affects the normal pace-maker. The new rhythm evoked following cardiac standstill is often irregular and appears to be elaborated outside the sino-auricular node(1). These effects may be potentiated by concomitant changes in the respiratory, urinary and central nervous systems(2).‡ The present experiments were initiated to determine the ECG patterns elicited by the intravenous administration of stingray venom.

Methods and materials. The venom of the round stingray *Urolophus halleri,* was used in all experiments. The venom was prepared by scraping the integumentary sheath(3) free of the animal's sting or caudal spine and macerating it with Ringer's solution in a cooled tissue homogenizer. Fresh stings were used in 12 preparations. Stings which had been immediately refrigerated at –20°C after being severed from the live animal were used in 14 preparations. Preliminary studies indicated that extracts of the venom from stings refrigerated for one week at –20°C were as lethal, or non-lethal, as extracts from freshly severed stings. The crude extract was centrifuged at 3000 rpm for 10 minutes. Each ml of the supernatant represented the venom from one, 4 cm sting. Electrocardiograms were made on 25 adult, pentobarbital anesthetized cats. Conventional limb, unipolar extremity and precordial leads were recorded on the electrocardiograms. In 10 cats, the systemic arterial pressure was recorded from the femoral artery. In 9 of the cats, intermittent artificial respiration was carried out by positive pressure breathing through a tracheal cannula. The remaining animals were allowed to breath normally. The venom extract was administered through the jugular vein.

Results. Abnormal electrocardiograms, prior to the injection of the venom, were obtained in 3 cats; these are not included in the data presented. There was no significant difference in the electrocardiograms of animals receiving the fresh extract as opposed to those receiving the extract from the refrigerated stings. The animals which received intermittent positive pressure respiratory stimulation tolerated the venom with fewer deleterious changes than those animals receiving no artificial respiratory stimulation. One ml of the extract provoked respiratory standstill of less than one minute duration in several of the uncannulated animals. The immediate ensuing respiration was slower, often irregular, and occasionally gasping in nature. Regular respiratory movements returned in 3 to 7 minutes following the injection of this amount of the venom. These appeared to be of lesser amplitude though adequate for survival. The anoxia was possibly reflected in the ECG pattern though similar patterns were elicited in the animals receiving artificial respiration. With larger doses of the venom the interruption of the respiratory cycle was longer. In 2 of the 3 cats receiving 3 to 4 ml, complete cessation of respiration occurred.

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‡ Unpublished data.

Two distinct effects on the heart were noted in the electrocardiograms: disturbances in rhythm and ischemic injury patterns. On injection of 1 ml of the venom the usual initial and immediate response was bradycardia with no apparent increase in the PR interval. Following this, and usually before the injection was completed, a prolongation of the PR interval was noted with characteristic patterns of first degree, second degree, and in some cases, third degree auriculo-ventricular block. Sinus arrest often followed the second degree block. Nodal, and less frequently, ventricular escape beats were recorded, usually with reversal to a normal sinus rhythm 20 to 30 seconds following the end of the injection period. In 3 of the cats receiving 1 ml of the extract and in 8 receiving 2 ml, there was an almost immediate ST, T wave change indicating an ischemic-type pattern. These changes persisted for 1 to 10 minutes then reverted to normal. On several occasions marked ST, T segment elevations were observed.

Where larger amounts of the venom were injected the patterns were abnormal and unpredictable. Seen frequently were all degrees of AV block, sinus arrest, interventricular block, decreased amplitude of QRS complex and patterns of various degrees of ischemia and injury. The rapid alteration of auriculo-ventricular conduction suggests strong vagal action, or more probably, direct toxic action on the auricles. The almost immediate ST, T wave changes indicate a direct toxic action on the ventricles. The late effects were too variable for interpretation. They were often influenced by the severe drop in systemic arterial pressure and anoxia.

Summary. Intravenous injection of stingray venom produced various changes in the ECG patterns of cats. The extent of the change depends, for the most part, on the amount of venom injected. Small amounts of the venom produce bradycardia with an increase in the PR interval giving a first, second or third degree auriculo-ventricular block. The second degree block is usually followed by sinus arrest. Reversal of the small dose effects occurs within 30 seconds following the end of the injection. Cats receiving larger amounts of the venom show, in addition to the PR interval change, almost immediate ST, T wave change indicative of ischemia and in some animals true muscle injury. These changes often persist for 10 minutes before reverting to normal. When fatal amounts of the venom are injected the pattern is abnormal and unpredictable. All degrees of auriculo-ventricular block, sinus arrest, interventricular block, decreased amplitude of QRS complex, and various degrees of ischemia and injury are seen. The rapid alteration of auriculo-ventricular conduction suggests that the venom has a direct effect on the auricles. The alteration in the ST, T wave suggests that the venom also has a direct effect on the ventricles. These experiments support previous observations(1).

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Changes in Plasma Concentration of Epinephrine and Norepinephrine With Muscular Work. (23561)

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Although it has long been known that adrenal medullary hormones are released during physical work, their interrelationship and the sequence of events leading to their release are not well understood. After discovery of norepinephrine as the postganglionic adrenergic nerve transmitter(1) and its role as an adrenal medullary hormone(2), it became obvious that many of the earlier experiments on the adrenal medulla failed to differentiate between epinephrine and norepinephrine. Consequently, the separate roles of epinephrine and norepinephrine during muscular work need to be established more clearly. A rise in catechol amine activity during muscular work was first demonstrated by Hartman(3) and, soon afterwards, verified by Cannon(4). Raab(5), using a non-specific chemical method, reported an increase in epinephrine-like compounds in human blood during exercise. More recently, von Euler and Hellner(6) showed an increased urinary excretion of both epinephrine and norepinephrine after prolonged work. Catechol amine concentrations in plasma probably give a more dynamic picture of transitory changes in sympathetic activity associated with work, than does excretion of these substances in urine. New technics for direct determination of the catechol amines in plasma are now available. Using these technics, we undertook to discover what changes occur in plasma concentration of epinephrine and norepinephrine in normal young men after moderate work (walking) and acute and chronic, severe work (running).

Methods. Six young, male volunteers (mean weight 73 kg) were used as subjects. They wore T-shirts, Army fatigue trousers, socks and sneakers. Breakfast was withheld. At 10 A.M. they began to walk on a treadmill at 3.5 mph on a 5% grade. After 30 minutes, they were immediately transferred to another treadmill, where they ran for 5 minutes at 7 mph on a 5% grade (2 men were able to run only 2.5 minutes). The run was an acute, exhausting stress for all. Blood was drawn just prior to the walk, within 2 minutes after the run, and at 15, 30, and 75 minute intervals following the run. These samples have been designated as "Control, 2, 15, 30, and 75 minutes." In a separate series on 5 of the same subjects, samples were taken within 2 minutes after the walk was completed. Plasma catechol amines were also determined on 3 well-trained athletes 5 to 8 minutes after running 18 miles in times varying from 1.8 to 2 hours. All blood samples* were taken from antecubital vein, with subject in reclining position, treated and analyzed for epinephrine and norepinephrine by the method of Weil-Malherbe and Bone(7,8) as reported by Gray and Young(9).

Results. It is apparent from Table I that there is a marked elevation (about 3-fold) in norepinephrine within 2 minutes after acute, severe work, with a drop to control levels within 15-30 minutes.

Epinephrine levels, however, are much less consistent than norepinephrine levels and show wide variation between individuals. The 2 minute, post exercise epinephrine levels vary from marked increase in one individual to no detectable change in 2 other subjects. The epinephrine levels, when elevated, return to normal in 15-30 minutes.

No change from control was found in epinephrine concentrations in the separate series after the walk, and in 4 of the 5 sub-

* The plasma removed for analysis was placed in lucite tubes, frozen in dry ice, isopropyl alcohol mixture, and shipped frozen to Biochemistry Dept., Walter Reed Army Inst. of Research, Washington, D.C., where the determinations were carried out.

TABLE I. Plasma Catechol Amine Levels* after Acute, Severe Work.

Subject	Control		2 min.		15 min.		30 min.		75 min.	
	E	NE	E	NE	E	NE	E	NE	E	NE
Ca	<.1	2.8	<.1	12.4					<.1	2.6
Mc	.1	2.2	1.4	7.9			.2	2.8	.1	2.1
De	<.1	3.2	.4	8.2	<.1	4.2	<.1	2.6		
Co	"	2.2	<.1	7.8	"	2.9	"	5.3		
Pi	.3	1.8	.6	6.1	.1	4.3	"	2.4		
Wi	.2	2.6	.7	6.0	.4	3.9	.3	3.6		

* All values are in μg/l.

Epinephrine values less than .1 μg are not detectable by this technic.

jects no change was found in norepinephrine values. In the fifth subject (De), the norepinephrine concentration changes from 2.1 to 4.5 μg/l.

Subjective evaluation of subjects by the authors with respect to motivation, muscular fatigue, difficulty in completing the test, and severity of emotional response suggests an association between increase in concentration of epinephrine in plasma and emotional stress; whereas muscular fatigue and difficulty in completing the work appeared associated with high norepinephrine levels in the plasma.

After prolonged, severe exercise, the plasma concentration of both catechol amines was elevated in 3 marathon runners following an 18 mile run (Table II). It seems likely that the values immediately after the run, *i.e.*, at times comparable to the acute experiments, were even higher than those reported in Table II.

Consistent elevation of plasma norepinephrine post exercise is in agreement with the work of Elmadjian(10) who found a marked increase in urinary excretion of norepinephrine and only a moderate rise in urinary excretion of epinephrine in active professional hockey players after a game. It is not surprising to find epinephrine and norepinephrine varying independently in their responses to exercise, since a selective release of epinephrine and norepinephrine has been shown to occur(11). Von Euler has suggested that the high plasma norepinephrine concentration following muscular work may result from a reflex release caused by vasodilatation of the blood vessels, in the muscle, with exercise(12). This would tend to cause a compensatory vasoconstriction. This hypothesis has gained support from the work of Goodall and Meehan(13) on the human centrifuge and is in accord with our results.

TABLE II. Plasma Catechol Amine Levels* after an 18 Mile Run.

Subject	D.T.		J.D.		W.G.	
	E	NE	E	NE	E	NE
Pre	<.1	3.0	<.1	2.5	<.1	2.5
Post	.2	8.7	.2	5.5	.3	4.7

* All values are in μg/l.

Epinephrine values <.1 μg are not detectable by this technic.

Summary. 1. Concentration of epinephrine and norepinephrine has been measured in human plasma after acute and chronic work. 2. Norepinephrine increases markedly with acute muscular work (about 3-fold) and returns to normal within 15-30 minutes after work. 3. Epinephrine response to acute muscular work shows wide variation between individuals, varying from marked rise to no detectable change. Epinephrine values, when elevated, also return to control levels within 15-30 minutes.

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Renal Glutaminase and Carbonic Anhydrase Activities in Potassium-Deficient Rats.* (23562)

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The kidney enzymes carbonic anhydrase and glutaminase are believed to play an important role in renal electrolyte excretion (1,2). An increase of renal glutaminase activity has been observed in rats after administration of NH_4Cl(3-6) and a reduction after administration of $NaHCO_3$ or $KHCO_3$(3,4,6). The carbonic anhydrase activity has been found to be reduced after NH_4Cl administration(3). On the other hand, both glutaminase activity(3,5,7) and carbonic anhydrase activity(3,7) appear to be elevated in potassium-deficient rats. The results of recently reported studies on dogs(8) indicated the possibility that levels of kidney glutaminase and carbonic anhydrase activities are influenced by the dietary anion and cation content and by adrenal cortical activity. Consequently, the present studies were conducted with (a) intact rats fed diets containing varied amounts of K, Na or Cl, with and without simultaneous administration of desoxycorticosterone acetate (DCA), and (b) adrenalectomized rats fed a control or a low-potassium diet, with and without simultaneous administration of DCA.

Methods. Young adult male rats of the Wistar strain, of a uniform age and weighing between 250 and 300 g, were placed on a control diet for a period of 7 days before the experimental periods of 14 days were begun. The control diet was the same as the one employed in previous studies(3,7). The experimental diets represented the control diet altered only with respect to the content of K, Na or Cl. The adrenalectomized rats fed the control and the low-potassium diets were allowed 0.25 M NaCl *ad libitum*. The intact and adrenalectomized rats given DCA received 2 mg DCA (Percorten, Ciba Pharmaceutical Products)/rat/day by subcutaneous injection. At the close of the dietary period, the animals selected for the kidney enzyme studies were killed by decapitation and exsanguinated. The kidneys were quickly removed and subjected to the enzymatic analyses according to the procedures previously described(7). The remaining animals were placed under sodium pentobarbital anesthesia for the collection of blood and skeletal muscle (gastrocnemius from both hind legs). The chemical analyses of plasma and muscle were carried out by methods previously reported (3).

Results. The results of the chemical analyses of plasma and skeletal muscle and of the analyses of kidney glutaminase and carbonic anhydrase activities of intact and adrenalectomozed rats are presented in Table I.

Kidney Glutaminase. With intact rats, the kidney glutaminase activity was found increased in animals fed the low-potassium diet, and, following administration of DCA to animals maintained on (a) low-potassium, (b) low-potassium and low-chloride, and (c) low-potassium, low-chloride and low-sodium diets (lines 3, 4, 6 and 8). On the other hand, reduced glutaminase activity was found when the animals were fed the following diets: (a)

* This investigation was supported by a research grant from N.I.H.

TABLE I. Average Electrolyte Content of Plasma and Muscle and Kidney Glutaminase and Carbonic Anhydrase Activity.*

Diet†	Plasma				Skeletal muscle				Kidney		
	HCO_3	Cl	Na	K	H_2O, g	Cl	Na	K	N, mg	Glutaminase, U	Carbonic anhydrase, U
	— mEq					mEq					
1. Control	27.8	106.6	156.3	4.5	324.3	5.1	9.5	45.7	31.9 ± 1.6	3040 ± 217	383 ± 49
2. " + DCA	37.7	98.6	154.6	3.2	326.7	5.1	15.3	37.9	32.4 ± 1.8	3244 ± 336	315 ± 50
3. Low K	29.5	104.8	156.9	3.2	321.7	5.0	13.5	37.5	32.3 ± .9	4984 ± 390	609 ± 17
4. " + DCA	42.5	91.7	159.6	2.5	332.4	4.9	17.6	32.0	31.0 ± .9	7836 ± 132	633 ± 21
5. Low K and low Cl	47.7	83.4	153.4	2.9	322.5	4.4	18.3	34.4	33.3 ± .5	2590 ± 230	620 ± 61
6. *Idem* + DCA	54.4	77.9	154.7	1.9	326.2	4.3	22.8	29.6	33.8 ± 1.5	5792 ± 287	644 ± 32
7. Low K, low Cl and low Na	31.1	102.0	151.9	3.7	319.8	4.7	10.1	45.3	34.3 ± .1	2416 ± 120	578 ± 57
8. *Idem* + DCA	33.1	102.6	150.9	3.0	322.0	4.8	13.9	38.3	33.7 ± .1	5179 ± 445	503 ± 46
9. Low Na and low Cl	30.9	103.2	150.5	5.1	324.0	5.0	8.6	48.6	32.8 ± .8	2257 ± 187	457 ± 18
10. *Idem* + DCA	32.0	105.8	151.1	5.0	331.3	5.2	9.0	47.5	33.0 ± .4	3243 ± 391	406 ± 21
11. Adrenal X and control	27.2	113.8	156.5	5.4	339.1	6.7	10.8	47.2	33.1 ± 1.2	2805 ± 255	379 ± 33
12. *Idem* + DCA	41.5	99.9	160.2	2.6	320.4	5.2	18.7	34.2	33.9 ± .2	2150 ± 211	256 ± 26
13. Adrenal X and low K	27.1	111.7	152.2	4.9	336.8	6.3	12.1	45.8	33.7 ± .4	3763 ± 291	190 ± 57
14. *Idem* + DCA	43.0	98.4	161.5	2.5	325.1	5.7	19.6	34.0	34.2 ± .6	3828 ± 322	224 ± 42

* Plasma values are expressed/kg plasma water; muscle values/100 g fat-free solids; kidney nitrogen as mg/g fresh kidney; and kidney glutaminase and carbonic anhydrase activities as units/g kidney N.

† Dietary period of 2 wk. Plasma and muscle values are based on 3 pairs of rats, while kidney data are based on 5 or 6 individual rats.

low-potassium and low-chloride, (b) low-potassium, low-chloride and low-sodium, and (c) low-sodium and low-chloride (lines 5, 7 and 9). A perusal of the data will reveal a lack of correlation between the glutaminase activity and the plasma bicarbonate concentration, or between the glutaminase activity and the concentrations of skeletal muscle potassium or sodium. In general, administration of DCA tended to raise level of glutaminase activity, particularly when the diet was deficient in potassium.

Adrenalectomized rats maintained on the control diet and allowed 0.25 M sodium chloride solution *ad libitum* exhibited a normal level of glutaminase activity (line 11). Glutaminase activity was reduced in adrenalectomized rats fed the control diet and administered DCA, and was increased significantly when the adrenalectomized rats were maintained on the low-potassium diet, with or without simultaneous DCA administration (lines 13 and 14). It is evident that in the absence of the adrenal gland kidney glutaminase activity can be increased, but the increase is smaller than observed in intact rats under comparable experimental conditions (compare lines 13 and 14 with 3 and 4).

Kidney carbonic anhydrase. With intact rats, carbonic anhydrase activity was found increased in animals fed a potassium-deficient diet, with or without simultaneous administration of DCA (lines 3 through 8). It will be noted that while the enzyme activity was increased in rats fed the low-potassium, low-chloride and low-sodium diet, with or without simultaneous DCA administration (lines 7 and 8), the carbonic anhydrase activity was not significantly increased in comparable animals maintained on a diet deficient only in sodium and chloride (lines 9 and 10). However, there appears to be a species difference regarding the influence of DCA on kidney carbonic anhydrase activity when given to animals on low-potassium or low-potassium and low-chloride diets. A reduced activity was observed in dogs(8) and a very slight increased activity in rats. There is no evident explanation for this difference. An inspection of the data will reveal that the car-

bonic anhydrase activity cannot be correlated with the plasma bicarbonate concentration (for example, compare lines 2 and 3) nor with the muscle sodium or potassium contents (compare lines 2 and 7). On the other hand, carbonic anhydrase activity was found reduced in adrenalectomized rats fed a low-potassium diet (line 13) and in adrenalectomized animals maintained on the control or the low-potassium diets and administered DCA (lines 12 and 14). It therefore appears that the same experimental conditions which result in decreased carbonic anhydrase activity in adrenalectomized rats lead to a significantly increased activity in the intact rats (compare lines 13 and 14 with 3 and 4).

Discussion. The results of the present experiments revealed that the kidney glutaminase activity and the carbonic anhydrase activity can be altered independently, by factors which are not clearly identifiable. Neither the glutaminase activity nor the carbonic anhydrase activity could be definitely correlated with the plasma bicarbonate concentration or with the composition of skeletal muscle in respect to potassium and sodium. Similar conclusions were arrived at by other workers(2,9) studying changes in kidney glutaminase activity. The present results indicate some relationship between dietary potassium and carbonic anhydrase activity, since with intact rats significant elevations of this enzyme were encountered only under circumstances of dietary potassium deficiency.

It has been previously reported(10,11) that the renal glutaminase activity is unchanged in adrenalectomized rats. Wilson and Seldin (11) found further that the adaptation of the glutaminase enzyme system following NH_4Cl loads was unimpaired in adrenalectomized rats. They therefore concluded that adrenal hormones are not required either for the maintenance or optimum activation of the glutaminase enzyme system in the rat. The results of the present experiments also indicated that kidney glutaminase activity can increase in the absence of the adrenal gland. However, such increases were of less magnitude than those observed in intact rats under comparable experimental conditions. In contrast, the carbonic anhydrase activity in adrenalectomized rats became reduced in experimental situations which in intact rats led to increased enzyme activity. Perhaps inadequate renal carbonic anhydrase activity may be a factor in the impaired ammonia production noted in adrenal-insufficient rats(12).

Summary. Kidney glutaminase and carbonic anhydrase activities were determined in intact and adrenalectomized rats maintained on normal and potassium-deficient diets, with and without simultaneous administration of DCA. The changes of enzyme activity could not be related to plasma or muscle electrolytes. The activities of the 2 enzymes were found unaltered in adrenalectomized rats. Experimental conditions resulting in increased glutaminase activity in adrenalectomized rats had the same effect to a greater degree in intact rats. Kidney carbonic anhydrase activity failed to increase in adrenalectomized rats under circumstances which in intact rats led to increased activity.

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Phospholipid Antithromboplastin Not Related to Sphingosine.* (23563)

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In recent papers(1,2), Hecht has reiterated a statement that the phospholipid anticoagulant studied by us(3,4) probably owes its activity to sphingosine.

The work described here tests this hypothesis by chemical reactions that destroy sphingosine or the phospholipid selectively. These reactions were brought about using mixtures made artificially from the phospholipid and sphingosine. By means of chromatography and the assay for antithromboplastic activity(4) it was possible to detect the undestroyed products. In addition, many differences between sphingosine and the phospholipid antithromboplastin, both chemical and biological are listed.

Materials and methods. The assay for anticoagulant activity has been described elsewhere(4). The clotting system contained 0.1 ml of human plasma, 0.1 ml of the solution to be tested, (or water in the control), 0.1 ml of human brain thromboplastin, 0.1 ml of 0.02 M calcium chloride solution. The test solution of the phospholipid was made using sodium desoxycholate as a solubilizing agent. The final concentrations of test substance and of sodium desoxycholate were the same. Sodium desoxycholate alone gave clotting times similar to those of the control, in the range of concentrations employed. The dl-sphingosine† was tested in aqueous solution without desoxycholate. Clotting tests were done in siliconized tubes at 37°. The meaning of the numerical activities assigned to the phospholipid fractions is described elsewhere(4). The unit of activity is the ratio of the clotting time of the test material to that of a standard at the same concentration of 1 mg/ml in a clotting system described above. The paper chromatography used Whatman No. 1 paper in a system of diethyleneglycol, n-butanol, and water (4:1:1, by volume) and the material was located by spraying with ninhydrin (0.5% in butanol saturated with water).

Results. The first experiment was based on the ready destruction of sphingosine by periodate(5-7).

Experiments were run in parallel using 200 mg of phospholipid antithromboplastin with and without the addition of 20 mg of dl-sphingosine.† As a control, 20 mg of sphingosine was treated separately. The experiments were carried out in aqueous solutions using 600 mg of sodium periodate (0.3 M) for periods from 45 minutes to 48 hours. Two different phospholipid fractions were employed of 200 and 180 units of activity per mg(4,8). In order to determine whether any sphingosine was left after the oxidation, the oxidation products were chromatographed on paper beside synthetic dl-sphingosine. Athough 5 μg of sphingosine could be detected on the paper with ninhydrin, no sphingosine could be found in the oxidation products, even when the entire product from 20 mg of sphingosine was put on the paper. In order to test for anticoagulant activity, it was necessary to remove the inorganic salts by dialysis. The 2 phospholipid preparations then had anticoagulant activity of 150 and 160 units/mg.

Preparations of the phospholipid anticoagulant were completely degraded by reduction with lithium aluminum hydride in ether, although sphingosine resists this treatment. This reaction was indeed utilized by Grob and Gadient(9) in their total synthesis of sphingosine. When a mixture of 200 mg of phospholipid and 100 μg of dl-sphingosine was reduced and the mixture was treated with just sufficient water to decompose the lithium aluminum hydride, and the ether was removed after drying over sodium sulfate, the product had no anticoagulant activity.

* Aided by a grant from the U. S. Public Health Service.

† The dl-sphingosine was kindly provided by Dr. D. Shapiro and by Dr. C. A. Grob and was obtained by total synthesis.

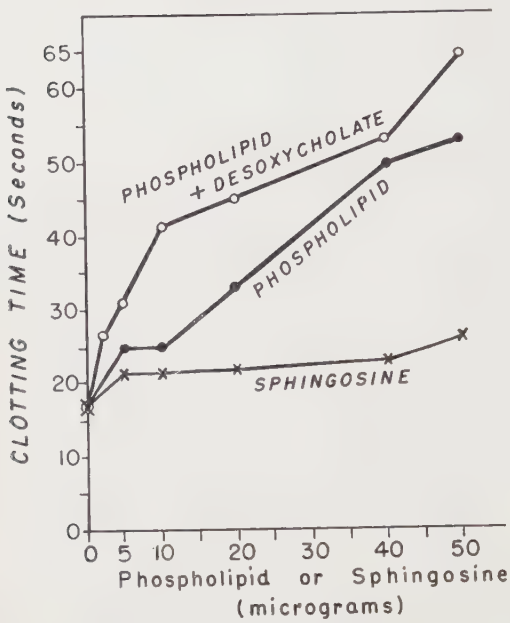

FIG. 1. Effect of phospholipid antithromboplastin fraction and dl-Sphingosine† (aqueous solution) on clotting time of activated, normal human, citrated plasma. Clotting system—see text.

Sphingosine was shown to be still present by paper chromatography of an amount corresponding to 16 micrograms of the original sphingosine. This paper chromatography was complicated by the effect noted by Marinetti's group(5), a slowing of the movement of the sphingosine in the presence of the products from the reduction of the phospholipid. This difficulty was overcome by reducing the phospholipid and then adding sphingosine to the reduction products to compare with the reduced mixture of sphingosine and phospholipid. The ethereal solution from the reduction of the phospholipid alone did not give a reaction with ninhydrin.

Comparison of anticoagulant activity. Sphingosine is relatively inactive (Fig. 1), in the activated clotting system used to assay the phospholipid, which measures the ability of the substance to neutralize the clot-accelerating effect of brain thromboplastin. This clotting test employs citrated human plasma, with which sphingosine forms a precipitate. The phospholipid antithromboplastin is, moreover, an active anticoagulant when injected into dogs(4). Unfortunately, the activity of sphingosine has not been studied *in vivo*.

Comparison of other chemical properties. Sphingosine forms a water-soluble hydrochloride, but the phospholipid antithromboplastin retains its activity when thoroughly washed with dilute hydrochloric acid(10). The phospholipid is destroyed by very mild treatment with alkali, while sphingosine is ordinarily prepared by release from its sulfate with alkali and resists alkali at 100° (5,11,12).

The purified phospholipid antithromboplastin has been studied by the paper chromatographic methods of Amelung and Böhm(13) and of Lea, Rhodes and Stoll(14). Dr. Marinetti of the University of Rochester has kindly studied its behavior for us in his solvent systems(15,16). The intact phosphatide has 2 components corresponding to phosphatidylserine and probably a lyso- or acetal-phosphatidylserine. After hydrolysis, serine was the only nitrogenous base found by paper chromatography in a variety of solvents. Cruder fractions, like the Folch fraction III (17), with anticoagulant activity of 160 units/mg, also contain phosphatidylethanolamine and some contaminating amino-acids. The amino-acids can be removed by the cellulose column method of Lea, Rhodes and Stoll(14), with complete retention of activity. It is of interest that when 100 μg of dl-sphingosine in chloroform solution is percolated through 13 g of moist cellulose, the sphingosine is completely retained. This is also true when the sphingosine solution is acidified(14).

Summary. Methods involving separate selective destruction of sphingosine and phospholipid antithromboplastin in mixtures of the two indicate that they cannot be the same. Differences in their chemical and biological properties also support the view that they are distinctly different anticoagulants.

We wish to thank Dr. C. A. Grob and Dr. D. Shapiro for their gifts of authentic sphingosine and Dr. Guido V. Marinetti of the University of Rochester for his assistance and advice.

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Inhibition of Azo Dye Carcinogenesis by Adrenalectomy and Treatment With Desoxycorticosterone Trimethylacetate.*† (23564)

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Symeonidis, Mulay and Burgoyne(1) reported that adrenalectomy or overdosage of intact rats with desoxycorticosterone acetate (DCA) prevented azo dye carcinogenesis. On the other hand Griffin, Richardson, Robertson, O'Neal and Spain(2) got just the opposite results. The former authors employed 4-dimethylaminoazobenzene (DAB) and subcutaneous implants of pellets of DCA while the latter workers used 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB), and subcutaneous injections of DCA in oil. The amount of steroid given per month by either group was approximately the same, making it highly unlikely that differences in methods of administration of DCA accounted for the divergent findings. Also, it seems highly unlikely that the use of different carcinogens could account for the discrepancies since the effects produced by carcinogenic azo dyes are qualitatively the same(3); 3′-Me-DAB is a more rapidly acting hepatic carcinogen than is DAB(4). During the past 5 years, we have been attempting to determine the effects of adrenal steroids on the course of azo dye carcinogenesis and have followed the work of the above authors with considerable interest. We find, as did Symeonidis *et al.*(1), that treatment of totally adrenalectomized rats with 11-desoxycorticosterones prevents azo dye carcinogenesis, but unlike these workers, we find that overdosage of intact rats with 11-desoxycorticoids fails to prevent the carcinogenic process. Furthermore, we find that the presence of accessory or regenerated adrenal cortical tissues inhibits the protective effect of adrenalectomy and hormonal treatment. The basis of these conclusions is reported here.

Methods and materials. Male rats of the Long-Evans strain weighing approximately

* Desoxycorticosterone trimethylacetate was supplied by Dr. Robert Gaunt, Ciba Pharmaceutical Products.

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TABLE I. Effect of Adrenalectomy and DCT Treatment on Body and Liver Weights of Male Rats Fed 3′-Me-DAB for 4 Months.

Group	Treatment	Body wt gain, g	Liver wt, g/100 g body wt	Liver wt, g	
				Non-nodular†	Nodular
		Mean ± S.E.			
1 a	Intact, food *ad lib*	1 ± 19* (6)	9.2 ± 3.4 (5)	10.5 ± .3 (5)	11.2 ± 12.1 (4)
b	" 10 g food/day‡	18 ± 8 (6)	8.2 ± 1.4 (6)	14.9 ± 3.1 (6)	4.4 ± 2.9 (5)
2 a	" food *ad lib*, 250 mg DCT	61 ± 11 (16)	8.1 ± 1.0 (15)	15.1 ± 1.0 (15)	7.9 ± 3.9 (9)
b	" 10 g food/day, 250 mg DCT	41 ± 9 (5)	8.7 ± 1.7 (5)	12.9 ± 1.8 (5)	8.4 ± 4.2 (5)
3 a	Adrx., food *ad lib*, 250 mg DCT	58 ± 4 (8)	4.1 ± .1 (8)	10.8 ± .5 (8)	None
b	" 10 g food/day, 250 mg DCT	68 ± 5 (3)	4.2 ± .2 (3)	11.8 ± .6 (3)	"
4	Subtotal adrx., 250 mg DCT (2 from fed group) (3 from *ad lib* group)	56 ± 9 (5)	5.2 ± .8 (5)	14.5 ± .7 (5)	§

* S.E. $= \pm \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

† Non-nodular refers to liver tissue other than dissectable, neoplastic nodules. In grossly tumorous livers, non-nodular tissue contained scattered lesions and tiny nodules.

‡ In groups 1 b, 2 b and 3 b amt of food was 15 g/day for 2 wk, thereafter 10 g/day.

§ Nodules too small to be dissected and weighed.

210 g initially were fed a semisynthetic diet, patterned after Price *et al.*(5), containing 0.058% 3′-Me-DAB for 126 days. The dietary ingredients were:

Dextrose	620	g/kg
Salt mixture (Wesson)	40	
Vit. test casein	240	
Corn oil	100	
Thiamin (Cl)	3	mg/kg
Riboflavin	1.5	
Pyridoxine (HCl)	2.5	
Ca pantothenate	7	
Choline (Cl)	30	
3′-Me-DAB	580	
Halibut liver oil	300	
Tetracycline-Vet	100	

The diet was similar to that used by others working on azo dye carcinogenesis except that it contained a higher percentage of casein. Also, an antibiotic, (Pfizer, Tetracycline-Vet), was added in hopes that it would reduce respiratory infections that commonly occur in rats fed purified diet containing azo dye. Sixty rats, all of which were fed the carcinogenic diet, were divided into 3 groups: group 1, 12 rats, served as intact controls; group 2, 24 intact rats, received desoxycorticosterone trimethylacetate (DCT); group 3, 24 adrenalectomized rats, received DCT. Each major group was subdivided into 2 subgroups as given in Table I. Desoxycorticosterone trimethylacetate (DCT), a long-acting adrenal-like steroid(6), was injected intramuscularly in 5 mg doses immediately following adrenalectomy. One week later, after the healing of incisions, the rats were injected with 70 mg DCT and placed on the carcinogenic diet. One month later, 75 mg of the steroid was given, followed with 50 mg at the beginning of the second and third months. Intact rats received DCT in a manner and time sequence similar to adrenalectomized ones; each treated rat received a total of 250 mg of the corticoid. The animals were sacrificed after they had been on the diet for approximately 4 months (120-126 days). At sacrifice, the animals were weighed and injected with a lethal dose of Nembutal. The blood vascular system was perfused with physiological saline; the liver was removed, examined, photographed and weighed. Small pieces of the left lateral lobe were removed for histological and enzyme studies. Nodules of neoplastic tissue, when present, were dissected, weighed and saved for nitrogen determinations. Histological, nitrogen, and enzyme studies will

TABLE II. Effect of DCT on Adrenal and Thymus Weights in Male Rats Fed 3'-Me-DAB for 4 Months.

Group	Treatment	Adrenal wt, mg/100 g body wt, mean ± S.E.	% change from control	Thymus wt, mg/100 g body wt, mean ± S.E.	% change from control
1	Intact control	15.5 ± 1.3 (11)		46.0 ± 3.4 (10)	
2	Intact, 250 mg DCT	11.0 ± .5 (20)	−29	42.4 ± 3.9 (20)	− 7.8
3	Adrx., 250 mg DCT			65.5 ± 8.8 (11)	+42.4
4	Subtotal adrx., 250 mg DCT	3.0 ± 1 (5)	−81	54.2 ± 5.4	+17.8

be reported later. The adrenals (when present) and thymi were weighed. In adrenalectomized rats the perirenal sites were examined for accessory cortical tissue grossly; when such tissue was found it was saved for histological study.

Results. Untreated intact rats feeding *ad lib.* showed the poorest weight gain. Treatment of rats, either intact or adrenalectomized, with DCT markedly improved weight gain and physical appearance. Hormonal treatment resulted in significant weight gain both in rats on a restricted and *ad lib.* diet.

None of the intact, non-treated rats died during the experimental period; two of the 24 intact animals treated with DCT, and eight of the 24 adrenalectomized rats treated with DCT, died.

Five of the 16 "adrenalectomized" survivors had adrenal cortical tissue. Such cases were classified as being subtotally adrenalectomized. As seen in Table II, the accessories were small, averaging 3 mg/100 g body weight. From previous work it is known that when rats are maintained for awhile on supportive therapy after adrenalectomy, appreciable numbers develop adrenal accessories and survive indefinitely(6).

Intact rats, including those on hormonal treatment had livers that were markedly enlarged (Table I) averaging about twice the weight of livers from adrenalectomized rats. Livers from intact rats fed the azo dye contained abnormal nodular growths, ranging in diameter from approximately 1 to 20 mm. The nodules were whitish in color and firm in texture. It was possible to dissect, by ordinary means, several nodules from most of these livers; such nodules, when examined histologically, were hepatomatous.

Adrenalectomized rats, without adrenal accessories, receiving hormonal treatment had normal appearing livers that were slightly larger than livers from intact rats fed a stock diet. Apparently the purified diet containing azo dye caused some liver enlargement, even in livers protected from the carcinogenic influence of the dye. The mean liver weight of adrenalectomized rats treated with DCT was significantly less than the mean liver weight of intact rats in either of groups 1b, 2a and 2b ($P < 0.05$ in each analysis).

Livers from subtotally adrenalectomized rats weighed, on the average, more than livers from totally adrenalectomized animals, but less than livers from intact rats fed azo dye. The carcinogenic process appeared to be delayed in livers from subtotally adrenalectomized rats.

As seen in Table II, DCT treatment in intact animals resulted in a significant decrease in mean adrenal weight, but had little or no effect on thymus weight. The mean thymus weight of treated adrenalectomized rats was significantly greater than that of treated or untreated intact rats ($P < 0.05$). Thymus gland weights of treated, subtotally adrenalectomized rats were essentially the same as those taken from intact controls. In other words, DCT treatment resulted in an expected atrophy (presumably due to ACTH suppression) of adrenal glands when present, but probably had little or no effect upon thymus weight.

Discussion. Results of these experiments indicate that 3'-Me-DAB carcinogenesis is inhibited by a combination of total adrenalectomy and treatment with desoxycorticosterone trimethylacetate. The presence of small amounts of adrenal cortical tissue allows the carcinogenic process to proceed, even when animals are treated with large doses of 11-desoxycorticoid. Preliminary data from our laboratory offer fragmentary evidence in

support of the report by Symeonidis *et al.* (1) that total adrenalectomy, without steroid treatment, prevents azo dye carcinogenesis. It is extremely difficult to keep the carcinogen-fed, untreated, adrenalectomized rat alive for several months; mortality is extremely high, even when saline is given as drinking fluid. Also, many of the "adrenalectomized" animals that survive the experimental period have adrenal cortical nodules with concomitant hepatomas; such factors complicate experiments and make it difficult to gather sufficient numbers to prove unequivocally that total adrenalectomy alone will prevent azo dye carcinogenesis. It may be, in our experiments, that the function of DCT was to keep the animal alive in the absence of other types of adrenal hormones, whereas, total adrenalectomy was the important factor in preventing carcinogenesis.

Controlling the daily intake of food was of little importance in these experiments since restrictive feeding or *ad lib.* feeding gave essentially the same results. During the first 4-6 weeks on the diet several adrenalectomized, DCT-treated animals died, but the survivors ate well, gained weight and were in good physical condition when sacrificed. Apparently DCT-treatment in either intact or adrenalectomized rats stimulated appetite; animals on the restrictive feeding regimen would have eaten more of the diet than was given. It is likely, in the *ad lib.* feeding experiments, that treated adrenalectomized rats ate more of the azo dye than did the intact controls. In other words, the amount of azo dye consumed by the rats with normal appearing livers was as great as in animals not protected from the carcinogenic process. Adrenalectomy (and DCT treatment?) apparently made it possible for the liver to reject the carcinogenic influence of 3′-Me-DAB.

Treatment of intact rats with DCT caused significant atrophy of the adrenal cortex but had little or no effect on thymus weight. Totally adrenalectomized rats treated with DCT had large thymuses, but this was probably due to lack of atrophy in the absence of the adrenals rather than to hormonal treatment. Such results are consistent with the findings of others who have studied adrenal and thymus weights in adrenalectomized rats and in intact and adrenalectomized rats treated with 11-desoxycorticoids(7). The finding that 29% reduction in adrenal weight induced by DCT had no influence upon the carcinogenic process is consistent with the finding that even small amounts of adrenal tissue support carcinogenesis. Total adrenalectomy is apparently the key factor in inhibiting azo dye carcinogenesis.

Summary. 1) Feeding of a semi-synthetic diet containing 0.058 percent 3′-methyl-para-dimethylaminoazobenzene (3′-Me-DAB) to intact rats for four months resulted in liver enlargement and development of large neoplastic nodules in 100 percent of the cases. 2) *Ad lib.* or restrictive feeding of azo dye were equally effective in inducing liver carcinogenesis. 3) Treatment of dye-fed, intact rats with large doses of desoxycorticosterone trimethylacetate (DCT), over a 4-month period, had no effect on liver carcinogenesis. 4) Treatment of dye-fed, totally adrenalectomized rats for 4 months with large doses of DCT prevented marked liver enlargement and inhibited development of macroscopic lesions and neoplastic nodules. 5) The presence of small amounts of adrenal cortical tissue allowed the carcinogenic process to proceed in rats fed azo dye and treated with large doses of DCT.

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Latent Viral Infection of Cells. V. Reappearance of Psittacosis Virus in Chick Embryo Tissues.* (23565)

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It has been demonstrated previously that cultures of minced chick embryo tissues, when kept in Hanks' balanced salt solution for more than 13 days, can be infected with psittacosis virus, but this tissue is not able to support the multiplication of the virus until the cells are stimulated by enriching the culture medium through the addition of embryo extracts or a synthetic tissue culture medium(1,2). This latent infection can be maintained for 11 to 12 days, *i.e.*, as long as the cells survive and infectious virus is not detectable during this period in the tissue culture fluid or in the tissue and thus is present as a noninfectious, latent infection(2). After stimulation of the cultures with nutrients, infectious virus reappears in the fluid, an effect which can be brought about whenever desired within the above-mentioned period. The present investigations were undertaken to see how rapidly the virus reappeared in such cultures when the nutrition of the cells was improved and to obtain further information about the nature of the latent infection and the growth cycle of the reactivated virus.

Material and methods. The psittacosis virus used was the 6BC strain which was passed in embryonated eggs via the yolk-sac route. The preparation of the stock virus has been described earlier(2). To infect the depleted tissue, the stock virus was diluted 1:1000 in Hanks' balanced salt solution (BSS), since it was found that a dilution of the stock virus 1:10 or 1:100 contained enough yolk material from the inoculum to stimulate the tissue, so that no latent infection could be obtained. The inoculum usually contained $10^{3.0}$ to $10^{4.0}$ LD_{50} per ml. The tissue culture technic has been described previously in detail(1,2), the only difference here being the use of Pyrex glass wool as a substrate for tissue growth instead of cellophane discs. The Pyrex glass wool was carefully washed in absolute alcohol and then rinsed several times with distilled water and once with triple-distilled water. It was then cut in circular discs to fit the bottom of a 10-ml Erlenmeyer flask and autoclaved. Minced chick embryo tissue was transferred to the Erlenmeyer flasks and to each was added 1.8 ml of BSS containing 0.025 ml of 1.4% sodium bicarbonate and 40 μg of streptomycin/ml. The cultures were incubated at 37°C and the BSS was changed completely after 24 hours and every 4 days thereafter. On the 13th day the cultures were inoculated with psittacosis virus and 24 hours later the fluids were replaced with a complete medium, *i.e.*, 10% beef embryo extract (BEE) in Hanks' BSS. Before changing to BEE and at varying intervals thereafter, 3 flasks were removed and were treated separately. The fluid was removed, diluted with an equal amount of sterile infusion broth and then tested in eggs for the presence of virus. The glass wool disc with the tissue was washed twice with 2 ml of infusion broth and then transferred to a mortar and ground, a procedure which has been shown not to inactivate the virus(2). 4 ml of infusion broth was added and the tissue suspension was titrated in eggs. Virus titers in culture fluids and tissues were calculated as the LD_{50} for 7-day embryonated eggs, according to the single-dilution method of Golub(3). Over a period of several years it has been noticed that the above-mentioned tissue culture technic does not work with chick embryo tissues during the hot summer months because the viability of the tissue is so decreased that after 13 days' depletion in BSS almost all of the cells are dead and there is

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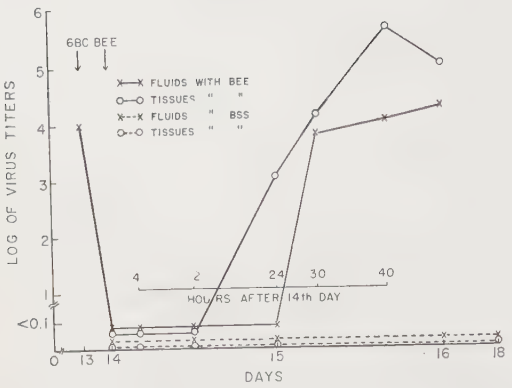

FIG. 1. Reactivation of latent infection with psittacosis virus following addition of BEE to tissue cultures.

no stimulating effect of BEE.

Results. Results of a representative experiment are shown in Fig. 1. In the present experiments it was found that no virus could be detected either in fluids or in tissue at 4 or 12 hours after stimulation of the cultures with BEE, though it had disappeared from the extracellular fluids. At 24 hours, infectious virus could be found in the tissue and sometimes in small amounts in the fluids also. At 30 hours, the virus was present in large quantities in the tissues and the fluids. After 40 hours, the virus could always be found in the fluids and tissues, although no virus was detectable in the fluids or tissues of cultures to which no BEE had been added. Virus appears earlier in the tissues and the titers remain higher than those of the corresponding fluids.

Discussion. In an earlier paper(2) the possibility that psittacosis virus was only attached to the deficient cells but had not entered the cells in the absence of factors present in BEE was discussed and data presented to refute it. Another possibility remained, namely, that the stimulation with BEE merely caused a liberation of absorbed virus from the depleted cells, but this was unlikely as no infectious virus could be detected even though the cells were homogenized to release any virus present(2). A prompt reappearance of virus would have supported such a theory, but, as the present investigations show, the reappearance of virus follows a normal growth curve. Girardi, Allen and Sigel(4) found that in tissue culture the titer of intracellular meningopneumonitis virus, which is closely related to psittacosis virus, starts to increase 20 to 30 hours after inoculation. Similar results were obtained by Buckley, Whitney and Rapp(5) using the fluorescent antibody technic, since inoculation of moderate amounts of psittacosis virus in tissue cultures resulted in intracellular appearance of psittacosis antigen in significant amounts in most of the cells within 20 to 24 hours. On the basis of these experiments it can be assumed that psittacosis virus in this experimental latent infection is present in the cells in the eclipse phase at a stage where the process of multiplication can easily be restored by the addition of nutrients such as amino acids(6), though the virus has lost its property of infectivity(2). The virus then readily resumes its normal growth cycle, even though this can be interrupted for as long as 12 days(2). The virus appears to exist in this latent infection in a ready though suspended state of animation.

Summary. Studies on the reappearance of psittacosis virus kept in a latent phase under experimental conditions in tissue culture indicate that the virus is in the eclipse phase and that it readily resumes a normal growth cycle following the addition of nutrients such as beef embryo extract.

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Phosphaturic Effect of Cortisone in Normal and Parathyroidectomized Rats.* (23566)

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In intact humans and dogs cortisone has been found to increase urinary phosphate excretion by depressing its renal tubular reabsorption(1,2). Since parathyroid hormone causes phosphaturia by a similar mechanism (3), the possibility that cortisone exerts its influence through the parathyroid glands must be considered. In favor of this possibility is the reported failure of urinary phosphorus excretion to increase in a cortisone treated patient with hypoparathyroidism and Addison's disease(4).

The investigation to be reported was designed to establish whether the phosphaturic effect of cortisone is mediated by the parathyroid glands.

Material and methods. Albino male rats weighing 150 to 200 g were used. Parathyroidectomy was performed surgically under ether anesthesia. Seven days postoperatively an elevation of serum inorganic phosphorus concentration to or above 11.5 mg % on a dietary intake which preoperatively had yielded mean levels of 8.7 ± 0.6 mg %, was taken as an indication of successful parathyroid removal. The experimental period was 3 days. The rats were kept in individual metabolic cages and, except as noted below, fed a known amount of ground, commercial rat chow.† Distilled water was given *ad libitum*. Ten mg of cortisone‡ was injected in one ml of saline intraperitoneally every day during the experimental period. In certain groups, phosphorus as a neutral Na_2HPO_4 and KH_2PO_4 solution was added to the food in an amount of 32 mg of phosphorus/rat/day. Urine of each individual rat was collected under thymol and pooled for the 3 day period. Blood was drawn by cardiac puncture under ether anesthesia at the end of each experiment. Serum and urinary phosphorus was determined by the method of Fiske and Subbarow(5). The urinary phosphate values are expressed as mg of inorganic phosphorus/100 g of mean body weight/24 hours. Mean body weight was determined by halving the sum of the weights at the start and end of the experiment. The parathyroidectomized animals were investigated by the cross-over experimental design. Thus some groups acted as controls and then received cortisone, others received cortisone first and were studied without treatment later. There was an interval of at least three days between periods of study.

Results are shown in Table I. In Section A it is evident that in intact animals serum inorganic phosphorus concentration is not significantly influenced by cortisone administration. Urinary phosphate excretion values, however, are about 60% greater in the presence of cortisone. In Section B the influence of parathyroidectomy as reflected in the higher serum inorganic phosphorus concentration is seen. Here as with the intact animals it is evident that cortisone promotes phosphaturia without significant effect on serum inorganic phosphorus concentration.

To test whether phosphate loading influences the cortisone effect, the data shown in the lower part of Section B, Table I were obtained. Phosphate loading in the parathyroidectomized rats was productive of great variability in the serum and urinary phosphorus values, which may have been due to irregular and incomplete intake of the load. In spite of this it is evident as in non-loaded

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† Purina Rat Chow, Ralston Purina Co., Chicago, Ill.

‡ Cortone Acetate, Sharp and Dohme.

TABLE I. The Effect of Cortisone upon Serum and Urinary Phosphorus in Intact and Parathyroidectomized Rats.

Section A: Intact rats						
Untreated			Cortisone treated			
SIP	UVP	C_p	SIP	UVP	C_p	Statistical evaluation*
1	2	3	4	5	6	
8.08	12.3	153	7.64	21.0	276	1 *vs* 4: $t = .67$ $p < .5$
9.68	9.47	98	8.09	24.6	304	
8.48	13.4	158	7.44	17.9	242	2 *vs* 5: $t = 4.12$ $p < .01$
7.72	8.13	105	7.16	16.7	234	
8.80	18.0	204	8.45	16.6	197	3 *vs* 6: $t = 4.96$ $p < .001$
8.45	12.0	145	8.65	22.5	260	
8.5 ± .67‡	12.2 ± 3.13	143.8 ± 38.7	7.9 ± .59	19.9 ± 3.3	252 ± 36.8	

Section B: Parathyroidectomized rats						
SIP	UVP	C_p	Study sequence	SIP	UVP	C_p	
7	8	9		10	11	12	
12.2	4.07	33.4	——→	13.6	11.1	81.6	7 *vs* 10: $\bar{m}\Delta = .99 \pm .5$ †
13.6	5.83	49.9	——→	13.7	9.4	68.6	$t = 1.93$ $p = < .1$
12.4	4.50	36.3	——→	12.5	7.6	60.7	8 *vs* 11: $\bar{m}\Delta = 4.6 \pm 1.65$
12.4	6.77	54.0	←——	15.6	14.0	90.0	$t = 2.78$ $p = < .05$
12.2	6.17	50.4	←——	13.2	9.5	72.0	9 *vs* 12: $\bar{m}\Delta = 28.5 \pm 4.77$
12.8	6.85	53.5	←——	12.8	9.7	75.9	$t = 5.98$ $p = < .01$
Phosphate loaded							
13	14	15		16	17	18	
12.3	20.2	149	——→	11.7	23.2	199	13 *vs* 16: $\bar{m}\Delta = .15 \pm 1.1$
14.6	26.5	163	——→	16.2	21.9	135	$t = 0.136$ $p = < .9$
18.7	37.2	178	——→	13.6	26.0	191	14 *vs* 17: $\bar{m}\Delta = 9.62 \pm 6.65$
11.7	15.7	135	←——	14.3	39.5	276	$t = 1.45$ $p = < .1$
11.2	15.2	135	←——	11.9	44.3	372	15 *vs* 18: $\bar{m}\Delta = 91.8 \pm 12.65$
12.9	17.8	138	←——	12.8	35.5	277	$t = 7.25$ $p = < .001$

SIP = Serum inorganic phosphorus; expressed as mg/100 ml. UVP = Urine phosphate; expressed as mg/100 g wt/24 hr. C_p = Phosphate clearance; expressed as ml/100 g wt/24 hr.

* Note that in Section A the comparison was made between 2 separate groups of 6 animals each. In Section B the comparison was made between values obtained on 2 occasions in the same animal; thus the degrees of freedom in computing probability in Section A were 10, in Section B, 5.

† ± stand. error. ‡ Mean ± stand. dev.

animals that the phosphaturia was augmented by cortisone. Statistically the increase of phosphaturia falls short of significance. However, if these data are viewed in terms of clearance which takes into account the large variability of serum inorganic phosphorus, the cortisone effect becomes significant.

One circumstance was encountered, that of starvation, in which the phosphaturic effect of cortisone was absent. This is evident in the data of Table II for cortisone treated and control parathyroidectomized rats given no food but allowed free access to water over the three day period of study. The large weight losses consequent to the starvation are indicated. Here it may be noted for comparison that the fed rats of Table I showed a mean weight gain, expressed as percent of starting body weight, of 1.6% ± 6.55 when no cortisone was given and a mean weight loss of 2.0% ± 6.7 during periods of cortisone treatment. Other measurements of urinary phosphate output not included in Table II corroborate the finding that in starvation cortisone fails to augment phosphaturia. It is of interest to compare phosphate excretion rates of starved animals of Table II with those of the fed but non-loaded parathyroidectomized animals of Section B of Table I. The phosphate excretion values of the starving animals are similar to those of fed animals treated with cortisone but are appreciably higher than those of fed, non-cortisone treated controls.

Discussion. It is evident from the data

TABLE II. Effect of Cortisone on Serum and Urinary Phosphorus in Starved Parathyroidectomized Rats.

Untreated				Cortisone treated			
		Urine PO_4				Urine PO_4	
Wt loss, %	Serum PO_4, mg %	mg/100 g wt/24 hr	clearance, ml/100 g wt/24 hr	Wt loss, %	Serum PO_4, mg %	mg/100 g wt/24 hr	clearance, ml/100 g wt/24 hr
16.8	15.1	12	79.6	21.8	15.6	16.3	104.5
23.8	13.0	15.6	120.0	20.6	13.8	13.6	98.5
28.8	13.5	16.7	123.7	15.2	13.7	14.7	107.2

presented that cortisone increases urinary phosphate excretion in both intact and parathyroidectomized rats. The phosphaturic action is thus clearly not mediated by the parathyroid glands. Inasmuch as the phosphaturia due to the hormone is unaccompanied by elevation of serum inorganic phosphorus concentration and cortisone is known to have but little influence on the glomerular filtration rate of the rat(6,7), it seems fair to surmise that, as in dog and man(1,2), the increased excretion reflects inhibition of renal tubular phosphate reabsorption.

The renal effect of the steroid occurring in the absence of a reduction in serum inorganic phosphorus concentration suggests that the hormone has other influences which prevent depletion of the extracellular inorganic phosphate pool. In this regard it is known that cortisone increases gastrointestinal phosphate absorption in the rat.§ It may also act both to divert phosphate from incorporation in new protoplasm as well as to effect its release from organic tissue complexes(8).

A relatively high rate of phosphate excretion refractory to administered cortisone was observed in the acutely starved, parathyroidectomized rats. It appears that starvation may elicit endogenous release of renally maximally effective amounts of glucocorticosteroid. This interpretation is supported by the hypertrophy of the adrenal zona fasciculata in the acutely starved guinea pig(9) as well as by the increased 11-oxycorticoid excretion of fasted human subjects(10).

§ Unpublished data.

Summary. 1) The effect of cortisone on serum inorganic phosphorus concentration and urinary phosphorus excretion has been investigated in normal and parathyroidectomized rats. 2) It has been found that cortisone exerts a phosphaturic action which is not dependent upon the presence of the parathyroid glands nor accompanied by elevation of serum phosphorus concentration.

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Relationship Between Endogenous Antidiuretic Hormone Activity and ACTH Release in Man. (23567)

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On the basis of certain anatomic and physiological observations, the antidiuretic hormone (ADH), vasopressin, has been postulated to be the neurohormone responsible for stimulating ACTH release(1-3). The observation that the intravenous administration of vasopressin in man is associated with a rise in plasma hydrocortisone is in agreement with this hypothesis(4-7). If ACTH release is produced by ADH, one would expect to see increased plasma hydrocortisone levels associated with endogenous ADH release. The present study is concerned with an evaluation of this postulated mechanism of ACTH release. The experiments were designed to answer the following questions: 1. Is the endogenous release of ADH associated with a rise in plasma hydrocortisone concentration? 2. Are increases in plasma hydrocortisone concentration associated with a concomitant release of ADH? Accordingly, a number of stimuli have been employed which are capable of producing an increase either in adrenocortical activity or in ADH activity.

Methods. Thirty-two experiments were performed in 8 male and 7 female normal subjects ranging in age from 18 to 28 years. All tests were begun at approximately 8 A.M. with fasting subjects recumbent in bed. In the studies conducted on males, urine was collected by voluntary micturition. In most of the studies conducted on females, an indwelling urethral catheter was used. Venous blood samples were obtained by means of an indwelling Cournand needle. In all studies carried out during a water diuresis, a total of 1600 ml of water was given by mouth in 4 divided doses over a period of 90 minutes. Urine osmolality was determined by the method of freezing point depression employing an Aminco-Bowman freezing point apparatus. Plasma hydrocortisone was determined by the method of Silber and Porter(8), as modified by Peterson, *et al.*(9). Plasma sugar was determined by the method of Nelson(10).

Results. Fluid Deprivation. Comparative observations on the effect of fluid deprivation and water loading were made in 6 subjects on 2 different days. On both test days fluid deprivation was maintained for 14 hours (6 P.M. to 8 A.M.) preceding the test. On one day fluid deprivation was continued throughout the test, while on the other day endogenous ADH release was suppressed by the administration of 1600 ml of water. During continued water deprivation the mean urine osmolality was 950 mosm./kg H_2O (range: 882-1018) at 8 A.M. and 824 (range: 684-904) at 12 M. That ADH suppression had occurred as a result of water loading was evidenced by the fact that the urine osmolality decreased from a mean of 828 (range: 553-1043) at 8 A.M. to a mean of 125 (range: 84-188) at 12 M. Fig. 1 shows the mean hourly plasma hydrocortisone and urine osmolality values from 8 A.M. to 12 M. on the 2 test days. Despite the persistent ADH activity associated with continuing water deprivation, the plasma hydrocortisone levels decreased in a manner similar to those ob-

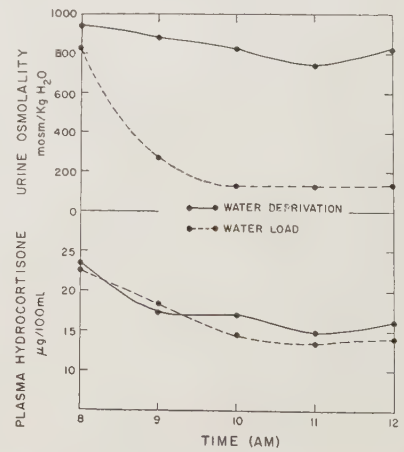

FIG. 1. Effect of fluid deprivation and water loading on plasma hydrocortisone concentration and urine osmolality for six normal subjects. Each plot is the mean for the 6 subjects.

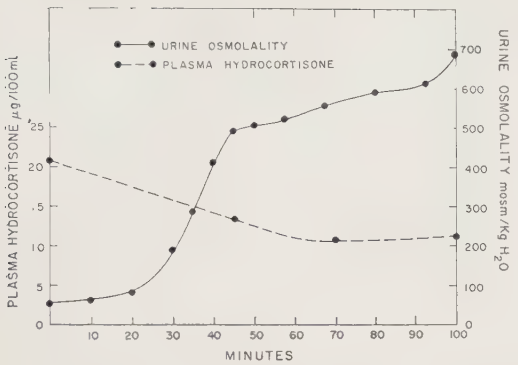

FIG. 2. Effect of 45-min. infusion of 5% saline on plasma hydrocortisone concentration and urine osmolality for three subjects during water diuresis. Each plot is the mean for the 3 subjects.

served during ADH suppression.

Hypertonic saline. Fig. 2 shows the effect of intravenous infusions of five percent sodium chloride solution on plasma hydrocortisone and urine osmolality values for three subjects during water diuresis. Five percent sodium chloride in volumes from 360 to 440 ml was administered over a 45 minute period. A rise in urine osmolality occurred in each case. The mean urine osmolality prior to the saline infusion was 57 (range: 55-60). During the saline administration the urine osmolality rose to a mean of 690 (range: 675-709). Despite this evidence of endogenous ADH release, the plasma hydrocortisone levels did not rise.

Nicotine. Intravenous injection of 3 mg of nicotine bitartrate (1 mg nicotine base) in 4 subjects during a water diuresis was followed by an increase in urine osmolality in all cases. Mean control urine osmolality was 71 (range: 61-77) rising to a maximum of 399 (range: 276-676) following the injection. Blood for plasma hydrocortisone determinations was obtained at 15, 30 and 60 minutes following the injection of nicotine. In 2 of the 4 subjects, plasma hydrocortisone concentration did not change despite the rise in urine osmolality.

Hand immersion in ice water for periods of 2 to 6 minutes was carried out in 8 subjects during a water diuresis with a mean baseline urine osmolality of 90 (range: 53-134). In 5 subjects urine osmolality increased to a concentration consistent with ADH release (mean: 321, range: 180-447). Two of these 5 subjects evidencing a rise in urine osmolality had no increase in plasma hydrocortisone concentration. One of the 3 subjects who had no increase in urine osmolality manifested a rise of 8 μg/100 ml in plasma hydrocortisone concentration. Thus in 3 of the 8 subjects evidence for simultaneous ADH and ACTH release was lacking.

Insulin-induced hypoglycemia. During water diuresis (mean urine osmolality: 66, range: 62-70) 5 subjects were given intravenous injections of 0.1 U crystalline insulin/kg body weight. This dose of insulin produced an average maximal fall in the plasma sugar of 54 mg/100 ml. A concurrent rise in urine osmolality and plasma hydrocortisone concentration occurred in only one subject. A rise in plasma hydrocortisone concentration occurred in 3 subjects without an associated increase in urine osmolality. The remaining subject manifested a rise in urine concentration of 330 mosm./kg H_2O with no associated rise in plasma hydrocortisone concentration. A composite of all the tests performed under conditions of initial water diuresis is shown in Fig. 5. It is apparent that there is no correlation between change in urine osmolality and change in plasma hydrocortisone concentration.

Discussion. In this study the 2 basic assumptions made are that increases in plasma hydrocortisone concentration and urine osmolality under the conditions of these experiments are valid evidences of ACTH and ADH release, respectively. ACTH release is pre-

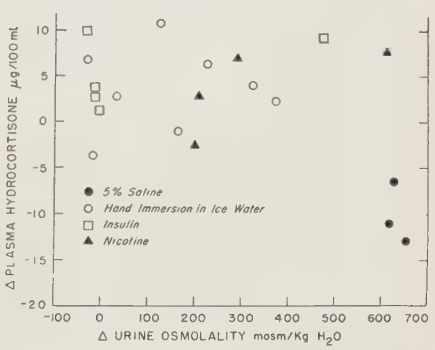

FIG. 3. Comparison of change in plasma hydrocortisone concentration and urine osmolality in four different test situations.

sumed if the plasma hydrocortisone concentration increases 3 μg/100 ml, 30 or more minutes following the test stimulus. This interpretation is based on observations of successive plasma hydrocortisone levels following intravenous injections of placebos in normal subjects under similar experimental conditions(6). A rise of 3 μg/100 ml plasma under the present experimental conditions is statistically significant ($P < 0.05$).

The use of urine osmolality as an index of ADH release under these experimental conditions is based on the following considerations. The average initial urine osmolality of 77 mosm./kg H_2O indicates that ADH secretion was greatly suppressed at the onset of all the tests(11) except fluid deprivation. Persistence of urine dilution of this degree is evidence for continuing suppression of ADH release. On the other hand, increases in urine osmolality, ranging from 115 to 654 have been interpreted as evidence of ADH release. Other factors, such as decreased glomerular filtration rate and increased solute excretion, are known to increase urine concentration in the absence of ADH(12-15). In the present study there was no evidence of an acute increase in solute excretion in any of the experiments with the exception of the intravenous administration of 5% saline. Urine concentrations greater than 600 mosm./kg H_2O observed during water deprivation and associated with the infusion of hypertonic saline during a water diuresis cannot be attributed to acute suppression of glomerular filtration rate(12). Thus the magnitude of the urine osmolality obtained during fluid deprivation and following hypertonic saline administration cannot be accounted for by any mechanism other than ADH release.

The increase in urine osmolality that results from hand immersion in ice water averaged 231 mosm./kg H_2O. The duration of the period of increased urine osmolality was at least 60 minutes in 3 subjects, 50 minutes in one subject, and 30 minutes in one subject. In view of the short duration of the hemodynamic response (<5 minutes) and the prolonged duration of the antidiuresis, such changes are interpreted as evidencing ADH release rather than a persistent reduction of glomerular filtration rate sufficient to cause changes of this magnitude.

In the insulin studies the persistence of a very low urine osmolality in 3 of the subjects indicates a continued suppression of ADH despite the rise in plasma hydrocortisone concentration.

The magnitude of the increase in urine osmolality following the injection of nicotine could conceivably result from hemodynamic changes in some of the studies. However, the duration of the response (at least 30 minutes) makes an ADH-mediated response the more likely interpretation.

Summary. 1) The hypothesis that ADH is the neurohormone responsible for ACTH release has been investigated in normal human subjects by simultaneous determination of urine osmolality and plasma hydrocortisone levels in various test situations. 2) The data indicate that in normal human subjects endogenous ADH release may occur without an increase in ACTH release. Furthermore, it has been shown that an increase in ACTH release may occur without evidence of ADH release. These observations fail to support the concept that endogenous ADH release stimulates the release of ACTH.

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Distribution of CO and Radiochromium in Blood and Tissues of Rabbit and Dog. I. Carbon Monoxide.* (23568)

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Comparative studies on methods for measuring blood volume have shown that the volume of distribution of carbon monoxide (CO) is 12 to 16% greater than that of radioactively tagged red cells in splenectomized dogs(1) and human subjects(2). The discrepancy has been attributed to accumulation of CO in extravascular pigments, chiefly myoglobin, as proposed by Haldane and Smith(3), but the sites of accumulation of CO have never been demonstrated directly. We have analyzed homogenates of various tissues from rabbits and dogs after simultaneous administration of CO and autogenous red cells tagged with radiochromium (Cr^{51}), using the radioactivity of each homogenate as an index of its blood content, and found the principal sites of CO accumulation to be red skeletal muscle and myocardium.

Methods. Fifteen ml of blood from each animal was tagged with Cr^{51}, and the cells were stored overnight in saline or their own plasma at 5°C(2). Tracheotomy was done under narcosis with sodium pentobarbital (Nembutal), and the airway was connected to a small rebreathing system for administration of measured amounts (15 to 100 ml) of CO gas(4). Ten ml of the animal's own Cr^{51}-tagged cells was injected into a vein. Twenty minutes later, blood was drawn from the heart or a large vessel into tubes containing Wintrobe's oxalate mixture. The animals were killed by injection of Nembutal or by exsanguination. Pieces of heart, spleen, lung, liver, kidney, skeletal muscle, brain, bone and bone marrow were excised, wiped dry and homogenized. The blood was analyzed for CO by the palladium reduction method of Wennesland(5), and 3 g portions of homogenates were analyzed by a modification of a procedure used for blood clots(6). Five ml of a 1% solution of pepsin powder‡ in 1 N H_2SO_4 was added to each portion of homogenate to accelerate liberation of CO. Six to 8 hours were needed for complete liberation of the gas and reduction of an equivalent amount of $PdCl_2$. After the iodometric titration was completed, each sample was brought to a total volume of 12 ml by addition of distilled water. Its radioactivity in counts per second was measured in a flange-type scintillation counter. A total of 4,096 counts was made. The counting error was $\pm$ 2%. The error of the analysis of CO in homogenates was assumed to be the same as that in the analysis of blood clots(6). To obtain the desired accuracy in the tissue analyses the animals were given relatively larger doses of CO and of tagged cells than are used in measurements of blood volume. The CO content in volumes % was compared to the radioactivity in counts per second to obtain CO:Cr^{51} ratios for each tissue homogenate (R_t) and for the blood of each animal (R_b). For each tissue homogenate the ratio, R_t:R_b, was calculated. With no

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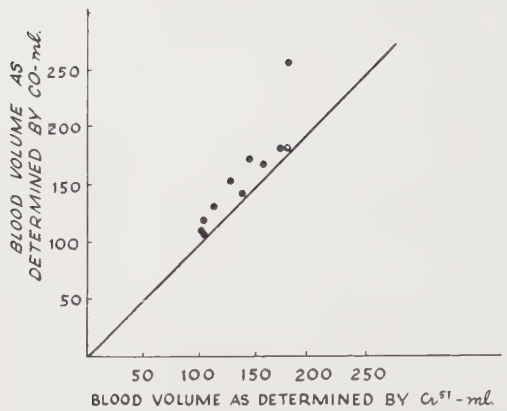

FIG. 1. Carbon monoxide-available space compared to Cr^{51} space in 11 rabbits. Mean difference is 13%.

loss of CO from the tagged circulating blood, the ratio R_t:R_b would be one. Extravascular accumulation of CO would give a ratio larger than one. The more CO and the less blood contained in a tissue, the larger this ratio would tend to be. A ratio less than one might be accounted for either by accumulation of Cr^{51} in the tissue or by loss of CO during the analytical procedure. However, the strong affinity of CO for hemoglobin and related compounds makes it improbable that gas is lost during the few minutes it takes to prepare the homogenates. Blood can be left in open bottles for weeks without appreciable loss of CO (see Table I). The volumes of distribution of CO and Cr^{51} were determined in the usual way(2).

TABLE I. CO Content, at Various Time Intervals, of a Specimen of Human Blood Stored at 5°C, Half of It in a Stoppered Bottle and Half in an Open Erlenmeyer Flask.

	Carbon monoxide conc., vol %			
	Storage time, days			
	0	30	37	40
Stored in stoppered bottles	3.27*	3.10*	3.25†	3.24*
Stored in open flask	3.30†	3.20†	3.17*	3.25†

* 2 N H_2SO_4 used for release of CO.
† 1 N H_2SO_4 plus 1% pepsin used for release of CO.

Results. Fig. 1 shows the volumes of distribution of CO and Cr^{51} determined simultaneously in 11 rabbits. The CO space exceeded the Cr^{51} space by an average of 13%. The difference is about the same as we found in human subjects, using the same technics(2), and as Root, Allen and Gregersen, using CO and P^{32}, found in splenectomized dogs(1).

Fig. 2 shows the results of analyses of blood and various tissue homogenates from 25 rabbits and 8 dogs, expressed as R_t:R_b ratios. Ratios greater than one were found consistently in skeletal and heart muscle. The scatter of the data for skeletal muscle can be accounted for largely by differences in the blood content of the various specimens. Generally, higher ratios were obtained in muscle tissue from dogs killed by bleeding

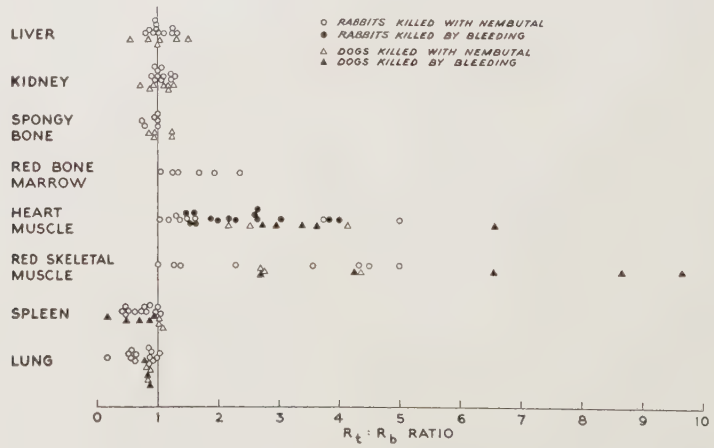

FIG. 2. R_t : R_b ratios of tissue homogenates from rabbits and dogs killed 20 min. after simultaneous administration of CO and Cr^{51}. R_t = ratio in tissue homogenate of CO content (vol %) to Cr^{51} content (counts/sec.). R_b = ratio of CO to Cr^{51} content of corresponding blood sample.

than from those killed with Nembutal. The influence of blood content on the quantitative result was emphasized by data from 2 experiments not illustrated in the figure, in which dogs were killed by viviperfusion with saline and exsanguination. Here, the $R_t:R_b$ ratios were 19.0 and 24.0 for skeletal muscle and 19.0 and 7.1 for myocardium. A modest excess of CO appeared in the red marrow of the rabbits; good samples of red marrow could not be obtained from the dogs. $R_t:R_b$ ratios of about one were found for liver, kidney and spongy bone. In both species, $R_t:R_b$ ratios below one were found in homogenates of spleen and lung, suggesting retention of the Cr^{51}-tagged cells in these tissues. The problem of Cr^{51} retention is discussed in part II of this study(8).

Discussion. The high $R_t:R_b$ ratios found in skeletal muscle and myocardium, and particularly in nearly bloodless muscle, support the suggestion of Haldane and Smith(3) that most of the CO leaving the blood combines with myoglobin. Data from 10 dogs permitted an estimation of the total amount of CO accumulated in the muscular system. The total muscle mass was taken to be a third of body weight(7). The quantity of CO in each sample of muscle homogenate was corrected for blood content on the basis of its Cr^{51} content. Assuming that the samples were representative of the entire muscle mass, it could be calculated that between 5.6 and 20.0% (average 13.5%) of the administered CO had accumulated extravascularly in muscle. Thus, making no allowance for the small amounts of CO in the myocardium and bone marrow, most or all of the discrepancy between the blood volumes measured with CO and with radioactively tagged cells can be accounted for by loss of CO to muscle. Data from 6 rabbits gave similar results.

Summary. 1. Carbon monoxide gas and autogenous red cells tagged with Cr^{51} were administered simultaneously to rabbits and to dogs. Blood and homogenized tissues from various organs were analyzed for radioactivity and for CO content. 2. The volume of distribution of CO was 13% larger than the volume of distribution of Cr^{51} in the rabbit. The discrepancy is the same as that found in man and the splenectomized dog. 3. CO accumulated in red skeletal muscle and myocardium in amounts sufficient to account for the discrepancy between distribution volumes. 4. Evidence was found pointing to slight accumulation of Cr^{51} in spleen and lung. Further studies of this phenomenon are described in part II of this report.

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Metabolic Fate of 11-Hydroxyandrostenedione-C^{14} in Human Subjects.* (23569)

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Introduction. It would appear that 11-oxygenated androgens are predominantly, if not entirely, of adrenocortical origin in human subjects. Romanoff, Hudson and Pincus isolated 11-hydroxyandrostenedione (Δ^4-androstene-11β-ol-3,17-dione, hereafter abbreviated Δ^4-11β-OH) from the adrenal vein blood of human subjects given ACTH(1). Salamon and Dobriner(2) isolated the same compound from the urine of a subject with myelogenous leukemia given ACTH. Twenty years ago Reichstein isolated adrenosterone and androstane-3β,11β-diol-17-one from adrenal cortical extracts(3). In addition, the 11-keto and 11β-hydroxy isomers of Δ^4-androstene-3,17-dione have been identified in blood perfused through beef adrenals(4). The latter isomer is probably also present in the adrenal venous blood of rats, sheep, and cats(5). All of these findings point to the probable adrenocortical origin of the 11-oxyandrogens. Produced in much lesser quantities (1-2 mg/day) than the 11-deoxyandrogens, the role of the 11-oxyandrogens in body metabolism, in both normal and abnormal conditions, remains obscure. The availability of C^{14}-labelled naturally occurring steroids has made it possible to study their metabolic fate in human subjects more accurately(6-15). Such studies have shown considerable differences among the various steroids in their rates of urinary, biliary and fecal excretions and in their physiological disposition. The biliary excretion plays a major role not only in the rapidity of urinary excretion of the metabolites of the steroids, but also in their ultimate disposition. The major aim of the present study was to investigate the metabolic fate of C^{14}-Δ^4-11β-OH and to compare the results with those of other steroids.

In this paper data are presented on the distribution of radioactivity in the bile, feces and urine following the intravenous injection of 4-C^{14}-Δ^4-11β-OH into 8 human subjects. In addition, the rates of clearance from the blood of the unconjugated steroids and their conjugated metabolites will also be presented. The identification of the various metabolites of the above steroid, as well as those of adrenosterone, is the subject of a separate communication.

Methods and materials. The 4-C^{14}-Δ^4-11β-OH was kindly supplied by the Endocrinology Study Section of the National Institutes of Health. The compound was chromatographed on paper in our laboratory and found to be more than 95% pure when the radioactivity was located with an Actigraph (Nuclear-Chicago) and the Rf compared with that of the standard steroid. One to 2 μc (5 μc/1.03 mg) were injected intravenously into 8 subjects: 2 normal males, 2 female subjects with bile-fistulas (T-tube drainage of bile), 3 normal female subjects without cancer and 1 female subject with cancer of the breast. All of the subjects were in good clinical condition and none had evident renal or liver dysfunction. None of the subjects had more than a mild anemia (hemoglobin concentration not less than 11 g %). The C^{14}-Δ^4-11β-OH was dissolved in 1-2 ml of absolute alcohol, diluted with 30 ml of physiological saline, and injected intravenously (I.V.) over a period of 1-2 minutes. Subject E.J. received I.V. 50 mg of adrenosterone (Δ^4-androstene-3,11,17-trione) in propylene glycol and human serum albumin just prior to the injection of the radioactive steroid(15). The 2 female subjects with T-tube drainage of bile had undergone cholecystectomies for chronic cholecystitis. The liver function tests in these patients were normal pre-operatively. The steroid was injected after each patient's condition had become stabilized, without fever or other

* This study was supported in part by grant from Natl. Inst. of Arthritis, N.I.H., U. S. Public Health Service.

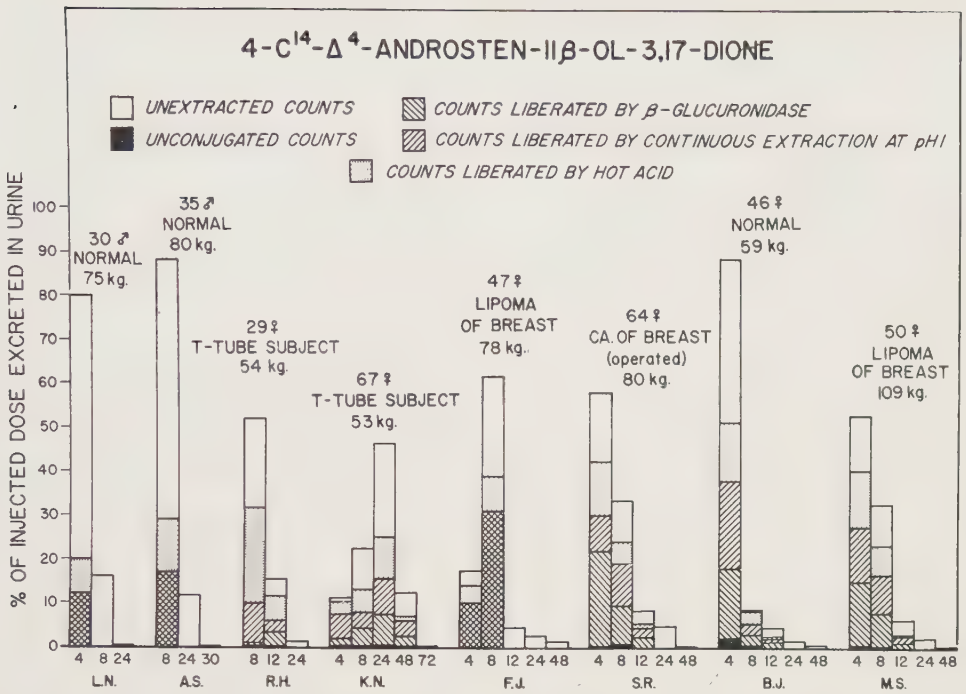

FIG. 1. Excretion of radioactivity in the urine following the inj. of C^{14}-Δ^4-11β-OH. Hydrolysis by β-glucuronidase of the urines of subjects L.N. and A.S. was incomplete due to low concentrations of the enzyme. Numbers on abscissa refer to the hour following inj. of the C^{14}-steroid when the collection was terminated. Totally blank columns indicate that no hydrolytic or extraction procedures were done on the urine specimens.

complications, after oral intake of fluids and food had become established, and 3 days or more post-operatively. The methods for collection and processing of urine, bile, feces and blood; the determination of radioactivity; the various hyrolytic and extraction procedures have been described in previous publications(12,15). Throughout this paper the word "unconjugated" will refer to steroid metabolites which are extractable with ether or chloroform before performance of any hydrolytic procedures; the "glucuronidates" to steroid metabolites which become extractable with ether or chloroform following β-glucuronidase hydrolysis; and the "sulfates" to steroid metabolites extracted continuously with ether for 48 hours at pH 1.

Results. The amount of radioactivity excreted in the urine following the I.V. injection of C^{14}-Δ^4-11β-OH averaged 95% for the group (range 79-106%), with the preponderant part of the radioactivity being excreted in the urine in the initial 8 hour period. In Fig. 1 is shown the urinary excretion of radioactivity during certain time periods following the injection of the radioactive steroid, as well as the amounts of steroid metabolites extractable following various hydrolytic procedures. Only negligible amounts of the injected steroid appear in the urine as unconjugated metabolites. Over 50% of the injected radioactivity was extractable following the various hydrolytic procedures (Fig. 1). When subject A.S. ingested 250 mg of adrenosterone and 2 μc of C^{14}-Δ^4-11β-OH, 61% of the radioactivity was recovered in the urine, of which 44% was extractable after β-glucuronidase hydrolysis and continuous extraction at pH 1, and 14% following hydrolysis by hot-acid.

Only small amounts of radioactivity were excreted in the bile (2.4 and 0.4%) of the two subjects with bile-fistulas and minute amounts were extractable following all hydrolytic procedures (0.3% and 0.08% of the total radioactivity).

TABLE I. Radioactivity Concentrations in Unconjugated (U), Glucuronidate (G) and Sulphate (S) Fractions of Plasma following the Injection of C^{14}-Δ^4-11β-OH. (Results expressed as % of inj. dose present in total plasma vol at various time intervals following administration).

	15 min.			30 min.			60 min.			120 min.			240 min.		
Subject	U	G	S	U	G	S	U	G	S	U	G	S	U	G	S
R.H.	3.2	23.5	.3	2.0	12.8	1.0	1.0	6.1	.1	.4	3.2	.1	.2	.9	.0
K.N.	5.4	11.6	.5	3.1	10.6	1.2	1.7	8.4	1.3	.6	5.5	.5	.2	2.9	.4
E.J.	4.4	14.1	1.5	2.4	16.7	.3	1.0	11.0	1.7	.9	6.1	.2	.4	2.9	.2
S.R.	3.1	10.8	2.4	2.8	12.1	.1	1.6	9.9	.7	1.0	6.5	.4	.8	4.3	.2
B.J.	3.1	19.8	.7	1.6	19.8	1.4	.8	13.6	.4	.4	7.1	.5	.1	2.6	.2
M.S.	4.1	22.1	.6	3.2	16.4	1.5	1.2	9.4	.5	.7	4.5	.4	.3	2.6	.3

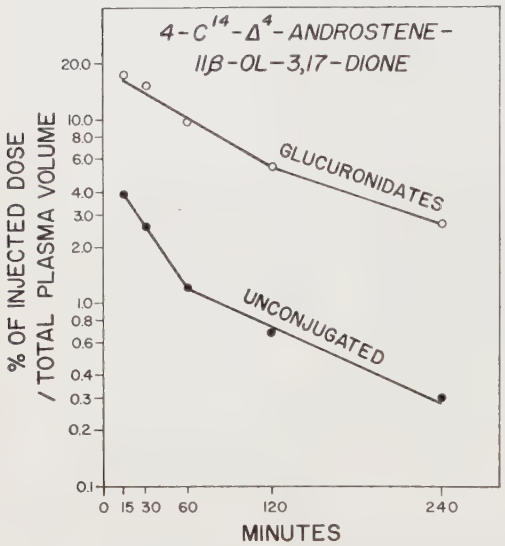

FIG. 2. Mean clearance of unconjugated and glucuronidated steroid radioactivity from plasma of 6 subjects following I.V. inj. of C^{14}-Δ^4-11β-OH. Note initial rapid rate and subsequent slower rate of clearance of the unconjugated radioactivity.

Negligible amounts of radioactivity were present in the stools of all the subjects.

The clearance of unconjugated radioactive steroids from the plasma of the injected subjects is shown in Fig. 2 and Table I. For the sake of comparison and uniformity, it was assumed that each subject's blood volume constituted 9% of the body weight (5% as plasma volume and 4% as the total red cell mass). It can be seen in Fig. 2 that the unconjugated steroids are apparently cleared at two separate rates with half-lives of approximately 30 and 80 minutes, respectively. Only negligible amounts (0.3%) of radioactivity were present in the unconjugated fraction of the plasma 4 hours following the injection. The glucuronidate fraction of the plasma contained 4 times as much radioactivity as the unconjugated fraction within 15 minutes following injection, and 10 times as much after 4 hours (Fig. 2 and Table I). The glucuronidate levels in the plasma declined rather rapidly during the initial 4 hours, decreasing from 17% at 15 minutes to less than 3% 4 hours after the injection. It is interesting to note that subject E.J., who received 50 mg of adrenosterone with the radioactive Δ^4-11β-OH, cleared the unconjugated and conjugated steroid metabolites at a similar rate as the other subjects.

Relatively small amounts of radioactivity were present in the sulfate fraction of the plasma (Table I). Negligible amounts of radioactivity were present in the plasma following hot acid hydrolysis or in the precipitated proteins. The amounts of radioactivity associated with the red blood cells are shown in Table II, and constituted, in general, a small fraction of the radioactivity injected when compared to that present in the plasma.

Discussion. From the data presented 2 findings concerning the metabolism of C^{14}-Δ^4-11β-OH are particularly of interest. First, more of the injected radioactivity is excreted in the urine following the injection of C^{14}-Δ^4-11β-OH than that seen following any other labeled steroid studies so far. In addition,

TABLE II. Total Radioactivity Levels Associated with the Erythrocytes following Injection of C^{14}-Δ^4-11β-OH.

Subject	% of dose present in total red cell mass				
	15 min.	30 min.	60 min.	120 min.	240 min.
R.H.	1.2	.7	.4	.1	.0
K.N.	.2	.5	.4	.4	.8
E.J.	1.2	.9	.8	.5	.5
S.R.	1.4	1.0	1.3	2.3	2.6
B.J.	1.6	.7	.5	.5	.4
M.S.	2.3	2.3	2.9	2.3	1.9

this radioactivity was excreted in the urine in a shorter period of time, essentially in the first 8 hours, than that required for any other C^{14}-steroid. This is in agreement with the findings of Bradlow, Hellman and Gallagher (13), who observed similar rapid excretion of radioactivity in the urine following the administration of C^{14}-Δ^4-11β-OH. Excretion of radioactivity in the bile or in the stools in the present study, was found to be the smallest when compared to that of any other steroid administered. These findings are particularly striking when compared to the rate of urinary excretion and the total radioactivity excreted in the urine, bile or feces following the injection of C^{14}-labeled estrone, estradiol, or progesterone(7,15,16). Even when compared to the urinary excretion of radioactive testosterone(6,12), cortisol or corticosterone(8,9,11), radioactive metabolites of Δ^4-11β-OH were excreted more quantitatively and more rapidly than was observed following the administration of any of the aforementioned steroids. These findings indicate rapid metabolism and excretion of metabolites of Δ^4-11β-OH. The lack of any substantial biliary excretion of radioactivity following administration of Δ^4-11β-OH probably plays an important role not only in the rapidity with which the radioactive metabolites are excreted in the urine, but also in the quantitative recovery of radioactivity in the urine. In previous studies it has been shown that substantial biliary excretion of steroid metabolites leads to slower excretion of radioactivity in the urine and/or lesser amounts of radioactivity in the urine due to involvement of the metabolites in a hepato-enteric circulation(12,16).

The authors have reported(14) the possible relationship between the binding of steroids to human plasma proteins and their biliary excretion. The most avidly bound steroids are excreted primarily in the bile (estrone, estradiol), whereas the least bound steroids are excreted largely in the urine (cortisol, cortisone). On the basis of the findings of the present study one would predict that Δ^4-11β-OH would be the least bound of the endogenous steroids studied, and, indeed, such is the case as reported previously (17).

The clearance of the unconjugated steroids from the plasma following the injection of Δ^4-11β-OH occurred at two different rates during the initial 4 hours. The rates were associated with metabolic pools whose half-lives were approximately 30 and 80 minutes respectively. Similar clearance rates have been observed following the administration of C^{14}-labeled progesterone(16), estrone(15), estradiol(15), testosterone(12) and corticosteroids(9,10). In the past, it has been postulated that the initial more rapid rate is related to the mixing of the radioactive steroid in the body pools, whereas the subsequent slower rate represents the true metabolic rate of the steroid(10). If this be true, it would appear that all these steroids have metabolic pools whose turn-over rates are not greatly different, although the pool sizes may be profoundly different.

Summary. 11-Hydroxyandrostenedione-C^{14} was administered I.V. to 8 human subjects: 2 normal males, 2 subjects with bile-fistulas, 3 females without and one female with cancer. The radioactivity was excreted in urine almost quantitatively within a very short period of time (8-12 hours). Only negligible amounts of radioactivity were found in the bile of the 2 subjects with bile-fistulas or in stools of all subjects. The unconjugated steroids were cleared from the plasma at 2 rates, a fast rate with a half-life of 30 minutes and a subsequent slower rate with a half-life of 80 minutes. The glucuronidate fraction of the plasma contained much higher levels of radioactivity than the unconjugated fraction within 15 minutes following the injection of the steroid and even more strikingly four hours following the administration of the radioactive steroid. The significance of these findings is discussed.

We wish to thank Dr. John Paine, Professor of Surgery, University of Buffalo School of Medicine, for his interest and for placing at our disposal patients with T-tube drainage.

Thanks are due to Miss Maria Foldes and Mr. Lawrence Beecher for technical assistance.

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A Method for Assay of Stuart Factor.* (23570)

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Stuart clotting defect was first described by Hougie, Barrow, and Graham in 1957(1). The name was applied after the surname of the patient in whom the deficiency was first identified, and who had previously been reported by Lewis, Fresh, and Ferguson(2) as a case of proconvertin deficiency. Although the patient showed the laboratory features of proconvertin deficiency, there was found to be cross correction of this patient with the original proconvertin deficient patient of Alexander, Goldstein, Landwehr, and Cook(3), indicating that the two patients were deficient in separate clotting entities. It was demonstrated, moreover, that this new factor was necessary not only for the action of blood thromboplastin but also for the action of tissue thromboplastin. It was present in serum, adsorbable by barium sulfate and Seitz filters, and relatively stable to heat and changes in pH. During early dicumarol therapy the concentration is relatively high, whereas later it is depressed. While in proconvertin deficiency the Russell viper venom time is normal(4), in Stuart deficiency the Russell viper venom time is prolonged.

The latter finding suggested that adsorbed beef plasma as prepared for the proconvertin test could be used as a substrate for the assay of Stuart factor if Russell viper venom was used as the thromboplastin. Consequently, the following experiments were performed using the proconvertin test as described by Owren and Aas(5) and modified by Adamis, Sise and Kimball(6).

Methods. The assay was performed in the same fashion as for proconvertin with the exception that Russell viper venom fortified with a lipoid extract of human brain (crude cephalin) was used as the thromboplastin. A chloroform extract of acetone dried human brain was prepared by the method of Bell and Alton(7). A dilution of one part of this crude cephalin preparation was made with 30 parts of veronal buffer pH 7.4, ionic strength 0.154.

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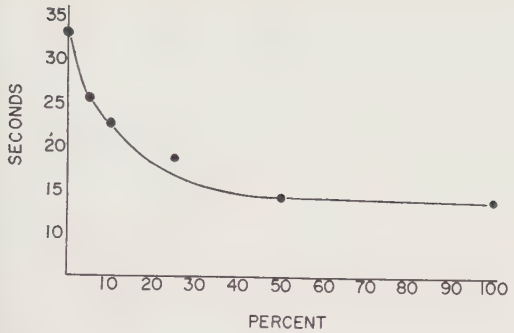

FIG. 1. Dilution curve showing clotting times in the assay system for various dilutions of pooled normal plasma in buffered potassium oxalate diluting solution. One hundred percent is a 1 to 10 dilution.

This diluted crude cephalin was used to dissolve the dried Russell viper venom (Stypven) in a strength of 1:40,000. The thromboplastin material thus prepared was frozen and stored at –20°C in aliquots sufficient for one day's use. The preparation is stable in the frozen state but deteriorates at room temperature sufficiently that refreezing for later use is not possible. A new dilution curve is necessary for each batch of viper venom cephalin mixture as well as reagent. Before recalcification of the mixture of reagent, 1:10 plasma dilution, and viper venom-cephalin mixture, it is allowed to incubate for 5 minutes. Conversion from clotting time of this mixture to percent normal is made on the basis of the standard dilution curve procedure.

Results. A sample dilution curve of a pooled sample of 5 normal plasmas is shown in Fig. 1. Although the clotting times do not show an ideally wide spread from the zero to 100% value, the reproducibility of the results have indicated that the test is accurate enough for ordinary purposes. Undoubtedly, Stuart factor is not completely adsorbed from the beef plasma reagent.

TABLE I. Clotting Times of the Assay System and Calculated Value of Stuart Factor in Various Plasmas and Sera.

Material	Clotting time, sec.	% normal
Normal plasma	14.0–16.5	100
Saline	30.3–42.5	0
Congenital proconvertin deficiency plasma	15.3	100
Congenital Stuart deficiency plasma	36	0
Congenital PTC deficiency plasma	16.2	80
Normal serum	15.1	100
Congenital Stuart deficiency serum	31.8	0
Congenital PTC deficiency serum	18.2	33

The clotting times and calculated value of Stuart factor for various plasmas are shown in Table I. We are indebted to Dr. John B. Graham for sending lyophilized samples of plasma and serum from the patient Stuart.

In Fig. 2 there is shown the response of Stuart factor and proconvertin to a dose of Warfarin, 300 mg intravenously, in 5 patients. The decrease in Stuart factor occurs gradually compared to proconvertin. This agrees with the findings of Hougie *et al.*(1).

The results indicate that the test is sensitive to Stuart factor, and insensitive to proconvertin and prothrombin. Owren and Aas(5) have previously shown the system to be insensitive to ac-globulin. It may be tentatively concluded that the test, therefore, is a measure of Stuart factor.

Discussion, Since in Stuart factor deficiency, the clotting time is prolonged with either

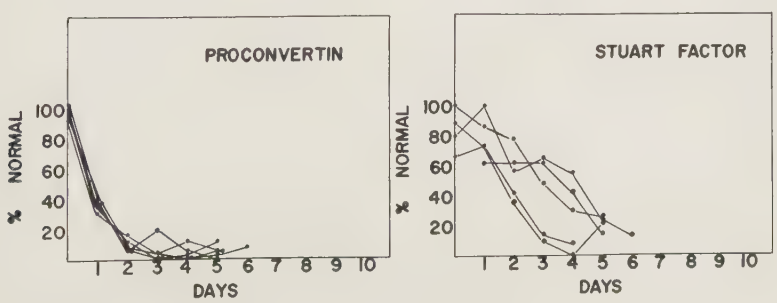

FIG. 2. Changes in proconvertin and Stuart factor after 300 mg of Warfarin intrav. in 5 patients.

tissue thromboplastin or viper venom, it is apparent that this factor is necessary for the action of both of these thromboplastic materials. It has been suggested that viper venom contains proconvertin or acts in place of proconvertin(4). It is also possible that there are two distinct systems for activation of the prothrombin conversion enzyme, one through the tissue thromboplastin route where proconvertin is necessary, and the other through the plasma thromboplastin route where proconvertin is not necessary. In this sense, proconvertin may be thought of as a true "cothromboplastin" but only for the tissue thromboplastin. Viper venom in this scheme appears to act in the same manner as plasma thromboplastin (platelets, antihemophilic globulin, PTC, PTA, and possibly other factors) for it does not require the presence of proconvertin for its normal action(8).

It seems probable from the findings that Hjort, Rapaport, and Owren's(9) method of assaying prothrombin by viper venom is also sensitive to Stuart factor.

Summary. The modification of Owren and Aas' test for proconvertin by substitution of viper venom-cephalin mixture for tissue thromboplastin is described as a method of assay for Stuart factor.

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Effect of Adrenocortical Hormones on Serum Proteins of Adrenalectomized Mice Infected with *M. tuberculosis*.* (23571)

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The electrophoretic distribution of serum proteins following experimental infections with *M. tuberculosis* has been studied in rabbits(1), guinea pigs(2-4), and rats(3). Also, the effects of adrenocortical hormone administration on serum protein patterns have been reported on rabbits(5,6), guinea pigs(7), rats(8), and dogs(6). It has been difficult to evaluate these results due to species variation, variation in severity and length of the infection, and differences in amounts and types of hormones administered.

This study was designed to investigate the effects of adrenocortical hormones on serum proteins of infected, adrenalectomized mice where there is no buffering effect on the intact adrenal gland, and to compare the effects of similar amounts of a glucocorticoid and a mineralocorticoid in this animal.

Materials and methods. Bilateral adrenalectomies were performed on 260 CFl albino mice. One hundred thirty of these mice and

* Hydrocortisone, desoxycorticosterone, and 2-methyl-9-fluorohydrocortisone were generously donated by Dr. Robert O. Stafford, Dept. of Endocrinology, Upjohn Co., Kalamazoo, Mich. Total protein determinations were performed by Jack A. Moorman and Jeanne LaCerte.

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TABLE I. Effects of Hydrocortisone (F), Desoxycorticosterone (DC), and 2-Methyl-9-fluorohydrocortisone (MFF) on Serum Proteins of Adrenalectomized Infected and Non-infected Mice after 5 Weeks of Infection and Hormone Administration.

Treatment	Total protein (g %)	Albumin*	Globulins*				
			$Alpha_1$	$Alpha_2$	$Alpha_3$	Beta	Gamma
Normal mean (45)†	6.07	54.87	4.06	9.73	5.16	20.21	5.97
" range (2σ)	5.25-6.89	45.05-64.69	3.28-4.84	8.29-11.17	2.20-8.12	10.27-30.15	1.33-10.61
Infected animals:							
Intact controls (8)	5.35	44.42	8.62	14.32	7.41	19.69	5.54
Adrenalectomized controls (3)	5.45	47.36	4.13	13.80	11.24	18.97	4.50
" + 500 γ F (8)	6.50	50.86	9.70	14.06	7.42	13.56	4.40
" + 500 γ DC (8)	5.50	47.44	5.18	13.34	10.54	18.62	4.89
" + 10 γ MFF (5)	5.50	52.30	4.30	8.61	9.26	19.92	5.61
Non-infected animals:							
Intact controls (9)	5.98	48.75	5.28	11.42	10.81	16.68	7.05
Adrenalectomized controls (10)	6.78	47.57	2.98	9.56	21.96	12.99	4.94
" + 500 γ F (6)	7.50	37.80	4.38	6.98	27.28	10.69	12.88
" + 500 γ DC (7)	6.72	42.36	3.80	11.65	15.11	19.71	7.37
" + 10 γ MFF (9)	7.08	28.49	3.18	10.03	38.45	11.97	7.88

* Protein fractions expressed as % area. † Total No. of animals/group.

20 intact mice were infected with *M. tuberculosis* var. *hominis* H37Rv by means of an airborne infection apparatus(9). The adrenalectomized mice were divided into 7 groups of 15-20 each the day after infection and treated as follows: oil-treated controls, 50 γ and 500 γ hydrocortisone/animal/day, 50 γ and 500 γ desoxycorticosterone/animal/day, a combination of 50 γ hydrocortisone and 50 γ desoxycorticosterone/animal/day, and 10 γ 2-methyl-9-fluoro-hydrocortisone/animal/day. The daily dose in each case was administered in 0.1 ml corn oil subcutaneously. The intact infected animals were treated only with oil. The 130 non-infected adrenalectomized mice were divided into the same groups, including a group of 20 intact, non-infected controls. The animals were bled to death by cardiac puncture. Approximately half were sacrificed at 2½ weeks and the remainder 5 weeks after injection or after the beginning of hormone administration. The blood drawn from each group was combined into one or 2 pooled samples due to the small amount obtained from each animal, and serum was obtained from the whole blood by centrifugation at 2,500 rpm for 10 minutes. Total protein was determined by a modification of the biuret method(10) and relative concentration of the serum protein fractions with the Spinco Model R Electrophoresis equipment consisting of a Durrum "hanging strip" type cell and a recording and automatically integrating densitometer(11). A normal range ($2\ \sigma$) was established from 10 pooled samples of blood from 45 untreated, intact, non-infected mice of the same age and weight (approximately 25 g). Degree of infection was determined by gross and histological examination of the lungs after autopsy.

Results. Over 90% of the normal determinations of each fraction fell within the normal ($2\ \sigma$) range (Table I). Since it was necessary to pool the blood from each experimental group, values which fell outside the normal range were considered to be significant changes.

After 2½ weeks of hormone administration all the non-infected mice, including both adrenalectomized and intact controls, showed a decrease in albumin and increase in the $alpha_2$ globulin. An increased $alpha_3$ globulin was observed in all of the groups after 2½ weeks of infection.

A summary of the results from 5 weeks of infection and/or hormone treatment is found in Table I. The results obtained with 50 γ hydrocortisone and desoxycorticosterone were similar to those demonstrated with the higher doses. In the non-infected animals, adrenal-

ectomy alone caused a slight decrease in the $alpha_1$ fraction and a marked increase in the $alpha_3$ globulin. With both doses of hydrocortisone, the total protein increased, the albumin decreased, and the $alpha_3$ globulin was slightly higher than in the adrenalectomized controls. The greatest decrease in albumin, an increase in the $alpha_3$ globulin, and a slight decrease in the $alpha_1$ globulin were caused by the 2-methyl-9-fluorohydrocortisone and the combination of hydrocortisone and desoxycorticosterone.

The infection with *M. tuberculosis* appeared to mask or possibly inhibit the effects of the individual hormones on the serum proteins. In the intact controls, the disease caused an increase in the $alpha_1$ and $alpha_2$ globulins and a slight decrease in the albumin.

All of the infected animals showed a marked degree of lung involvement both grossly and histologically. There was no correlation between severity of the disease and serum proteins as the high doses of hydrocortisone and desoxycorticosterone and the 2-methyl-9-fluorohydrocortisone caused the greatest degree of infection.

Discussion. There has been considerable variation in the reported effects of the adrenocortical hormones on the serum proteins depending on the species of the animal treated and length of time and level of administration of the compounds. Hoch-Ligeti and Irvine(8) reported elevation of albumin and total protein and decrease in gamma globulin in rats with large doses of hydrocortisone and cortisone, and no change with desoxycorticosterone following 5 days of administration. Cortisone has been shown to cause an increase in gamma globulin and decrease in albumin in guinea pigs after 15 days(7), but Snell and Nicol reported a decrease in gamma globulin in guinea pigs after 2 weeks of cortisone treatment(12). No changes, however, were observed in rabbit serum proteins after 30 days administration of cortisone and hydrocortisone(5,6). In dogs, a marked elevation of $alpha_2$ globulin was observed after cortisone and hydrocortisone treatments for 3 weeks (6). It is not too surprising, therefore, that the changes in serum proteins observed with hormone treatments in mice do not agree with those reported in other animals.

There are also considerable variations in the serum protein changes with *M. tuberculosis* infections in different species and with different strains of bacilli. An elevation in beta globulin and decrease in albumin has been reported in rabbits(1), while an increase in gamma globulin and decrease in albumin has been observed in guinea pigs(2,4).

It is felt that these results present some interesting possibilities for future study. To our knowledge, there has been no previous report of a disease inhibiting the effect of hormones on serum proteins and this observation alone warrants further investigation.

Summary. Following administration of varying amounts of hydrocortisone, desoxycorticosterone, and 2-methyl-9-fluorohydrocortisone to adrenalectomized non-infected mice and adrenalectomized mice experimentally infected with *M. tuberculosis,* changes in serum proteins were determined by a paper electrophoretic technique. An increase in total protein was observed with hydrocortisone; a decrease in albumin and an increase in $alpha_3$ globulin occurred with hydrocortisone, high doses of desoxycorticosterone, a combination of hydrocortisone and desoxycorticosterone, and 2-methyl-9-fluorohydrocortisone; and an increase in gamma globulin resulted with a high dose of hydrocortisone. The individual effects of the hormones on serum proteins were not apparent in those animals infected with *M. tuberculosis.* All of the animals, except those treated with 2-methyl-9-fluorohydrocortisone, showed an increase in $alpha_2$ globulin while only the high dose of hydrocortisone produced a decrease in albumin.

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Modification of Antibody Response in X-irradiated Rats by Injection of Spleen Homogenates. (23572)

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Sublethal doses of x-radiation are capable of inhibiting antibody response in experimental animals if antigen is given before or after irradiation(1,2). This inhibition can be reversed by lead shielding of the spleen or appendix during irradiation even if the spleen is removed soon afterward(3,4). Injections of homogenates of spleen and bone marrow have been shown to accelerate recovery from the hematopoietic damage caused by total-body x-irradiation(5-7). It was demonstrated recently that this protection by cell transfer is characterized by a "recolonization" process in which the injected cells proliferate and substitute for some of the animal's own tissues that were destroyed by x-rays (8,9). There has been relatively little study of the effects of heterologous and homologous post irradiation cell transfer upon the immunological process. The present work was undertaken to study antibody response of rats receiving homologous spleen cell injections up to 24 hours after 500 r total-body x-irradiation and stimulated from 3 to 48 hours later with an intravenous dose of sheep erythrocytes. Other investigators have attempted to restore or protect the immune mechanism of experimental animals by injection of various materials following irradiation. Of these, Jaroslow and Taliaferro have been able to produce considerable protection in rabbits by means of injection of whole spleen cells and of HeLa and yeast extracts when the material is given *mixed with the antigen* 24 hours after irradiation(10). The present experiments, on the other hand, have always involved separate administration of spleen cells and antigen from a *few hours to 24 hours* apart at different intervals after irradiation. A previous study, with a somewhat similar pattern, is that of Smith and his associates(11) in which spleen or bone marrow cells were given immediately after x-irradiation, immunization was carried out from 1 to 7 weeks later, and the circulating antibody titer was studied on the day of peak response. Under these conditions no difference in the rate of recovery of the antibody-forming capacity was noted between "protected" mice and mice receiving x-irradiation alone. *Spleen cells* were transferred into the irradiated animals in this study because of the demonstrated importance of the spleen in antibody formation in the rat. Under these circumstances, splenectomized animals form only a very small amount of circulating antibody(12). Rats injected intravenously with a particulate antigen show characteristic cellular changes in the spleen which appear to be closely correlated with the appearance of circulating antibody(13). These changes can be inhibited by a variety of treatments known to depress antibody formation(2,14,15).

Materials and methods. Young adult Sprague-Dawley albino rats were used throughout. Irradiation was administered by means of a General Electric Maxitron thera-

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TABLE I. Types of Treatment Administered to Rats Receiving 500 r of Total-Body X-irradiation. Several different combinations of the indicated variables were used in 9 experiments.

Time of inj. of spleen cells or erythrocytes (days after total body x-irradiation)	Sheep erythrocytes (1.0 ml of a 0.25% suspension given intrav.)	Spleen cells (2×10^8)												
			Harvested after antigenic stimulation (days)						Homogenization method*					
		Normal	1	2	3	4	5	6	1	2	3	4	Washed	Unwashed
0		x		x	x	x	x	x	x	x	x	x	x	x
1	x	x	x		x	x		x	x					x
2	x								x					x
3	x								x					x

* Homogenization methods: 1—Crushing in a porcelain mortar with pestle in small amt of Locke's solution. 2—Homogenization by hand with Potter type homogenizer. 3—Homogenization by motor driven Potter type homogenizer followed by straining through a U.S. Standard Sieve No. 100, 0.149 mm, Dual Mfg. Co., Chicago, Ill. 4—Cells forced out of the spleen by inj. Locke's solution into the organ after removing it. All cell preparations were filtered through a double layer of gauze and then aspirated through a gauge 28 needle to eliminate connective tissue strands and large cellular aggregates which may cause embolization of pulmonary vessels.

peutic machine. Irradiation factors were: 250 KVP, 30 ma., with 0.5 mm Cu and 1 mm Al added filtration. Target distance was 95 cm. The animals were irradiated in groups of 12 in a circular aluminum box with perforated top, each animal being housed separately in a small section. The box was rotated slowly during exposure. Each rat received a total of 500 r at the average dose rate of 29.4 r per minute. Irradiated rats received one injection of spleen homogenate containing 2 x 10^8 cells and one intravenous injection of 1 ml of 0.25% suspension of sheep erythrocytes at varying times up to 3 days after irradiation. Spleen homogenates were prepared from "normal" animals and from rats that had received an earlier intravenous injection of 1 ml of *S. typhi* vaccine. Several methods of preparing spleen homogenates were tried. It became apparent, however, that the most effective method of preparation was crushing the spleen in a mortar. Therefore, only this technic was used in the later experiments. Cells were counted in a hemocytometer after dilution in Hayem's solution. Smears of the original preparations were stained with methyl green-pyronine and percentage of different cell types was determined. Homogenates of "normal" spleens were examined as stained smears and contained a mixture of small lymphocytes, macrophages and primitive reticular cells. Homogenates of spleens from stimulated animals contained all of these cell types and a large proportion of the immature pyroninophilic cells ("antibody forming cells") implicated in antibody formation(13). The percentage of "antibody forming cells" increased from 10% of the total on the 2nd day to 50% on the 4th day after antigen injection and returned to 5-10% on the 6th day. In one experiment, a group of rats was also given the spleen cells in 3 divided doses which totaled 2 x 10^8 cells. In another experiment, spleen cells were given intraperitoneally. Table I summarizes the various types of treatments that were used in a number of different combinations. Rats were bled for antibody determination at 4, 6, 12, and 18 days. Hemolysin titrations were carried out as described by Cannon and coworkers(16). The double dilution method was employed starting with a serum dilution of 1:15. The last tube showing complete hemolysis was read as the end point rather than employing the more critical 50% hemolysis end point because only large differences in antibody-forming capacity were being considered (*i.e.*, some or none).

Results. The best protective effect on the mechanism of antibody formation was always observed in animals receiving spleen homogenates prepared by crushing the spleen with mortar and pestle. Homogenization by hand or by a motor driven homogenizer with subsequent straining through a #100 screen, or collection of cells by forcing Locke's solution in the spleen with a needle and syringe yielded preparations which, although effective, protected to a lesser degree than cells from

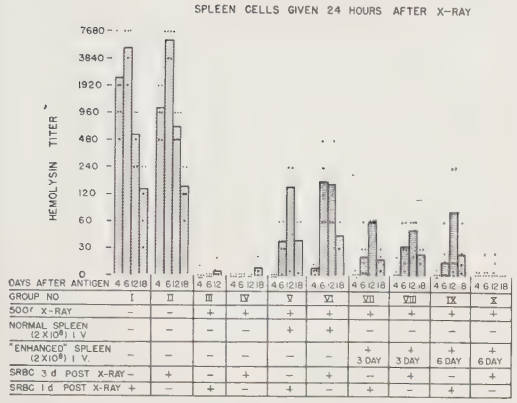

FIG. 1. Hemolysin titer in serum of rats receiving 500 r total-body x-irradiation and given spleen homogenates 24 hr after x-ray.

crushed spleens. A certain toxicity was observed in the homogenates. Washing with Locke's solution abolished toxicity almost entirely but also decreased the protective effect. Fractionating the cell infusion into 3 equal portions or changing the injection route from intravenous to intraperitoneal resulted in less protection.

The protective effect of cells from normal spleens as well as from spleens stimulated by antigen injection 3 or 6 days earlier is shown in Fig. 1. Normal rats stimulated with an intravenous injection of a particulate antigen will reach a peak in the titer of circulating antibody about the sixth day after injection. A delay in reaching the peak is apparent in the irradiated, spleen-injected rats as compared with normal control animals in the present series of experiments. Usually this peak is not reached until the 12th day after injection.

Fig. 2 indicates that essentially the same results are obtained by using normal spleen cells and cells taken from spleens stimulated with another antigen 2 to 6 days earlier. A substantial protection of the antibody-forming mechanism appears to be afforded by injection of either type of spleen cells, although the peak of circulating antibody is always considerably lower than in normal rats. It is apparent also that spleen cells collected during an active antibody response do not protect the antibody-forming mechanism of rats given 500 r of total-body x-irradiation to an extent greater than normal spleen cells. This is true regardless of the time of cell collection relative to antigen administration to the cell donors.

Discussion. Injections of homogenates prepared from "normal" spleens or from spleens of rats stimulated antigenically 6 days earlier may be somewhat more effective in restoring the antibody-forming mechanism of the irradiated rat than cells from the spleen of rats stimulated 2 to 5 days earlier. It appears definite that cells engaged in antibody synthesis are not more effective than those from "normal" spleens. The fact that the antibody response of x-irradiated, spleen-cell injected rats is not as great as that of normal animals may be the result of insufficient time for the transferred donor cells to recolonize completely, insufficient number of cells transferred, or other factors. In most instances, the appearance of the peak titer has been delayed beyond the usual time of 6 days after antigen injection. This delay can be interpreted as the time necessary for the injected cells to exercise their effect on the antibody-forming mechanism.

It should be noted that while the injection of spleen homogenates restores at least partially, the antibody-forming mechanism in the x-irradiated rat, a similar type of injection has been reported to be ineffective in the mouse (11). Comparative studies between the rat and the mouse during active antibody response and after x-irradiation may help to explain these differences.

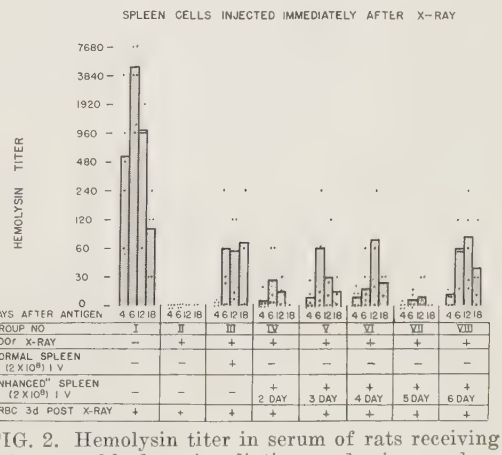

FIG. 2. Hemolysin titer in serum of rats receiving 500 r total-body x-irradiation and given spleen homogenates immediately after x-ray.

Summary. Antibody formation, which is inhibited by 500 r of total body x-irradiation into the rat, can be restored partially by spleen homogenates injected soon after irradiation. No striking difference in the degree of recovery is observed when spleen cells from rats stimulated earlier with a different antigen are used in place of "normal" spleen cells.

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Comparative Lipotropic and Lipide Phosphorylating Effects of Choline, Betaine, and Inositol.* (23573)

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It has been concluded that lipotropic action is more specific than stimulation of lipide phosphorylation since evidence showed that those substances which exert a marked lipotropic action also stimulate the formation of phospholipides, but that other substances stimulating phosphatide turnover may not necessarily cause a lipotropic effect(1). The lipotropic effect of choline, betaine, and inositol under specific dietary regimes is well known(2). However, the present study is, to our knowledge, the first to compare the lipotropic and phospholipide turnover actions of these compounds under identical experimental conditions. These conditions include the feeding of low protein-low fat and low protein-high fat diets to the experimental animals.

Methods. Male albino rats of Sprague-Dawley strain weighing 141 ± 43 g were divided into 2 groups. *Group I:* Ninety-nine rats, 26 of which served as controls, were maintained on a 5% casein-5% fat diet(3) or a 5% casein-32% fat diet(4) for a duration of 3 weeks. The animals were fed *ad libitum.* At the end of the dietary regime, the rats were stomach-tubed with a single dose of lipotropic agent (0.4 mM in 1 ml H_2O). All animals were then injected intraperitoneally with 1 ml of physiological saline containing 4 μc of P^{32} as NaH_2PO_4. Six hours later the animals were killed by decapitation. The liver was removed, rapidly weighed, and

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TABLE I. Effect of a Single Dose* of Choline, Betaine or Inositol on Total Liver Lipides and Lipide Phosphorylation.

Lipotropic agent	No. of rats	Total lipides, g	Relative specific activity
	5% casein—5% fat diet		
None	11	.84 ± .37	.186 ± .038
Choline	11	.59 ± .15	.282 ± .050†
Betaine	11	.59 ± .28	.230 ± .073‡
Inositol	10	.76 ± .38	.193 ± .034‡
	5% casein—32% fat diet		
None	15	2.06 ± .74	.255 ± .028
Choline	12	1.68 ± .96	.374 ± .057†
Betaine	14	1.44 ± .67	.284 ± .057
Inositol	15	1.61 ± .66	.251 ± .049

* 0.4 mM administered by stomach tube. No. preceded by ± are stand. dev. Test of significance was applied to difference between means of controls, saline, and experimental values. The P probability for chance occurrence of this difference was:
† <.01; ‡ <.05.

divided into 2 fractions. From one fraction, acid soluble phosphorus was removed by extracting with 10% TCA containing 0.4 M $MgCl_2$(5), and radioactivity and total P(6) were determined. The other fraction was covered with alcohol, and the lipides extracted with alcohol-ether and purified with chloroform(7). Radioactivity(7), phosphorus(6), and the weight of total lipides were determined on aliquots of the chloroform solution. As a measure of phospholipide turnover, specific and relative specific activities(8) were calculated. *Group II:* 59 additional animals were used to study the lipotropic actions of choline, betaine, and inositol when 100 mg/rat of each of these compounds was supplemented daily to a 5% casein-5% fat and to a 5% casein-32% fat ration(3,4) for 2 weeks. Fifteen of the rats served as controls. At the end of the dietary regime, all animals were injected intraperitoneally with P^{32}, and after 6 hours the animals were sacrificed by decapitation. The liver, after being rapidly removed and weighed, was divided into an acid soluble and phospholipide fraction for analyses as described above.

Results. The effects of single doses of choline, betaine and inositol are summarized in Table I. To evaluate the statistical significance of the results, the t test of significance (9) was applied to the difference between the means for control and experimental values. Choline exerted a pronounced increase in phosphatide turnover in animals maintained on either diet, whereas equal molar doses of betaine and inositol demonstrated an increase in the rate of phospholipide synthesis in rats receiving the 5% casein-5% fat diet. This increase was considerably lower than that observed for choline under these experimental conditions. It is also apparent that the phospholipide turnover rates in animals receiving the 32% fat diet was higher than that of those receiving the lower percentage fat diet. Although there was a decrease in liver fat in all the treated animals of Group I, the reduction was not significant over the control rats.

Only the total lipide data (Table II) from Group II are reported, since dietary experiments of this nature (in which smaller amounts of the active substances are ingested in divided doses) tend to show little change and considerable variation in the rate of synthesis, even though a slight change in the rate may cause a significant difference in tissue composition over a period of time(3). Choline and betaine produced an equally effective lipotropic response at the dietary dose levels used when supplemented to either diet. However, inositol produced this response only in the low protein-low fat diet.

Discussion. These results are in agreement with the evidence presented by workers who have conducted separate studies of these com-

TABLE II. Effect of Dietary* Supplements of Choline, Betaine or Inositol on Total Liver Lipides.

Lipotropic agent	No. of rats	Total lipides, g
	5% casein—5% fat	
None	7	.49 ± .13
Choline	7	.32 ± .08†
Betaine	7	.33 ± .05†
Inositol	6	.33 ± .09‡
	5% casein—32% fat	
None	8	.90 ± .65
Choline	8	.28 ± .05†
Betaine	8	.32 ± .07‡
Inositol	8	.75 ± .38

* 100 mg/rat/day. No. preceded by ± are stand. dev. Test of significance was applied to difference between means of controls and experimental values. The P probability for chance occurrence of this difference was:
† <.02; ‡ <.05.

pounds(2). Our study supports the concept that lipotropic action is more specific than that of lipide phosphorylation. Under those dietary conditions which led to a marked reduction in total liver lipides by a lipotropic agent, a corresponding increase in phospholipide synthesis was observed when the agents were administered as "acute" massive doses by stomach tube. Inositol, on the other hand, failed to exert any lipotropic response supplemented to the high fat diet and the administration of the "acute" single dose to animals on this diet caused no increase of the phospholipide turnover rate.

The equal effectiveness of all 3 agents in reducing the total lipides on the low casein-low fat diet is thought, on the basis of the recent work by Young *et al.*(10), to be due to the high dietary dose levels employed. At lower doses, however, these investigators reported the betaine to choline ratio necessary to produce a given fat level to be 3:1.

The order of effectiveness of single doses of the agents, in regard to lipide phosphorylation, under either dietary condition employed was the same, *i.e.*, choline > betaine > inositol. This greater stimulation by choline might well be expected, since choline itself is a part of the lecithin molecule. The results indicate that the transmethylation mechanism involved when a single dose of betaine is administered, is not nearly so effective as the administration of an equal molar dose of choline, even though the number of methyl groups available is the same. A reason for the low stimulation of phospholipide synthesis by inositol may have been the fact that inositol phosphatides represent a small percentage of the total liver phospholipides(11). Therefore, any increase in this portion would correspondingly have a small effect on the overall phospholipide turnover rate as measured by our methods.

Summary. The lipotropic and lipide phosphorylating effects of choline, betaine, and inositol were compared in rats maintained on low protein-low fat or low protein-high fat diets. All 3 agents (100 mg supplements/rat/day) were equally effective in causing a significant reduction of total lipides over controls in animals maintained on the low protein-low fat diet. Only choline and betaine supplements caused a substantial reduction of total liver fat in rats maintained on the high fat diet. Phospholipide synthesis was increased when choline, betaine, or inositol was administered as a single dose (0.4 mM) to animals maintained on the low protein-low fat diet. The order of effectiveness upon turnover rate was choline > betaine > inositol. Both choline and betaine, when administered as single doses, stimulated lipide phosphorylation in rats on the high fat diet, but inositol did not.

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Inhibition of Rat Cholinesterases by Tritolyl Phosphates.* (23574)

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Triorthotolyl phosphate has been of considerable interest to pharmacologists because of its ability to produce flaccid paralysis in fowl and in some mammalian species. The neurotoxic mechanism is obscure, and at present cholinesterase inhibition is not believed to be involved(1,2). It has been established that the pure compound does not inhibit cholinesterase *in vitro* but does inhibit plasma cholinesterase in rabbits on intravenous injection(3) and in fowl(4). The effect on erythrocyte cholinesterase of mammals has not been investigated previously, so far as we know. In this paper we report results of studies on inhibition of rat cholinesterases after intraperitoneal injection of 3 isomers of tritolyl phosphate.

Procedures. Triorthotolyl phosphate (TOTP), trimetatolyl phosphate (TMTP) and triparatolyl phosphate (TPTP) were obtained from the Eastman Organic Chemicals, Rochester, N. Y. Infra-red and mass spectroscopy indicated 99% purity, with the remaining 1% free of possible inhibitory agents. No ortho-groups were present in the samples of TMTP and TPTP, and the TOTP contained no pyro-groups. Male rats of the Long-Evans strain were used. For single-dose studies, their weights ranged from 85 to 110 g; for repeated-dose studies, the initial weights ranged from 120 to 150 g. The rats were randomized into groups for treatment, and were withdrawn from the repeated-dose experiment by random selection. *Single injection.* Groups of 5 rats were given 2 ml/kg of TOTP intraperitoneally and decapitated after intervals of 1, 12, 24, 48, and 168 hours. Groups of 5 rats were given 2 ml/kg of TMTP and TPTP intraperitoneally and decapitated after 24 hours. Groups of 5 uninjected control rats were sacrificed with the experimental groups in each case. TOTP and TMTP were given undiluted, while TPTP was given as a 10% emulsion in distilled water. The activity of cholinesterases in the erythrocytes, plasma, and brain was determined by the method of Frawley(5) and calculated as percentage with reference to the control groups. *Repeated injection.* The effect of repeated sublethal doses of TOTP was studied by injecting 2 groups of 10 rats with 0.1 and 0.5 ml/kg of TOTP intraperitoneally, thrice weekly, as 20 and 95% suspensions in propylene glycol, respectively. Control rats received repeated injections of 0.04 ml of propylene glycol, an amount equal to the largest quantity given the experimental animals. After sixth injection (on twelfth day), 5 rats given each level of TOTP and 5 controls were sacrificed for determination of cholinesterase activity in blood cells, plasma, and brain. Sections of liver, spinal cord, and sciatic nerve were retained for histologic examination. The remaining 5 rats in each group were sacrificed 10 days after the sixth injection, for cholinesterase determination and histologic study as above.

Results. Single injection. After a single intraperitoneal injection of 2 ml/kg TOTP, cholinesterase activity became increasingly less, to reach the lowest recorded value at 48 hours (Table I). At all intervals after the first hour, the erythrocyte enzyme was least active. Signs of acetylcholine accumulation were first seen 24 hours after injection, and were more severe after 48 hours. These signs were seen only in individuals with cholinesterase activity of less than 20% in the brain, erythrocytes, or both. They consisted of diarrhea, champing of teeth, muscle tremors including shaking paws, and occasional tear formation.

Rats sacrificed one hour after injection

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A preliminary report was presented before the Society of Pharmacology and Experimental Therapeutics, April, 1955, at San Francisco.

TABLE I. % Active Cholinesterase in Rat Tissues after a Single Intraperitoneal Injection of 2 ml/kg TOTP.

	Enzyme source		
Hr after inj.	Erythrocyte	Plasma	Brain
1	90.7	66.3	88.0
12	30.2	41.9	38.5
24	14.2	38.6	19.5
48	4.0	31.7	10.8
168	44.8	69.8	48.2

showed no gross abnormalities. A slight greasy appearance of the abdominal organs indicated incomplete absorption of the TOTP. Rats sacrificed at 12 hours had swollen livers with white mucoid material along their edges. Unabsorbed TOTP was still present in the cavity. In rats sacrificed at 24 and 48 hours there was a white mucoid material along the edges of the swollen livers, but no observable TOTP remained in the cavity. Seven days after injection, swelling was still present in the livers of half the rats, and the lobes were adherent. Small patches of white mucoid matter appeared to be contained within the liver tissue. The other rats of the group showed no abnormalities.

Microscopically, the animals showed a peritonitis of varying degrees of chronicity, depending on length of survival. The livers showed no parenchymal changes.

There were marked differences in the effects of the 3 isomers 24 hours after injection. TOTP lowered cholinesterase activity of all 3 tissues significantly (Table I) while TMTP significantly lowered only the plasma enzyme (60% of normal). TPTP had no significant effect on cholinesterase. TMTP and TPTP as well as TOTP had been completely absorbed by this time. No abnormalities were seen in rats injected with TMTP, and none in those injected with TPTP except for a few nodules scattered throughout the abdominal cavities of 2 rats.

Repeated injection. When repeated sublethal injections were given, TOTP again inhibited the true cholinesterase to a greater extent than the pseudocholinesterase. After 6 injection of 0.1 ml/kg, the cholinesterase of the erythrocytes was only 27% active while that of the plasma was 67% active; that of the brain was 47% active. With a 5-fold increase in the dose, the activity of cells or plasma was not significantly less than with the lower dose, but that of the brain was halved. None of the animals showed clinical signs of cholinesterase inhibition, presumably because the decrease did not pass the critical point. All of these animals had swollen livers with small white mucoid areas on the surfaces. Rats receiving the larger dose had in addition, small semi-solid lumps scattered through the omentum. The control rats were essentially normal.

The rats sacrificed 10 days later still had significantly lowered true cholinesterase values. The erythrocyte cholinesterase of the rats receiving 0.1 ml/kg was 67%, the brain was 55% active, and the plasma was up to normal, 97%. At the higher dose of 0.5 ml, the erythrocyte and brain values were somewhat lower, 53% and 38%, while the plasma was 80% active. These figures show that the activity of the brain enzyme is the slowest to be recovered, and suggest that it may be the most profoundly affected. These rats showed no clinical signs of cholinesterase inhibition, and no lesions except for swollen livers with patches of white mucoid material.

Microscopically, many of the livers exhibited a foreign-body reaction, a local effect of the compound which was not seen in the propylene glycol controls. The liver parenchyma was normal in all cases, and the spinal cords showed no difference between experimental animals and controls.

Discussion. It has been widely stated that TOTP is a specific inhibitor of pseudocholinesterase, and does not inhibit true cholinesterase. This belief apparently stems from a report of Mendel and Rudney(6) which stated that rats fed on the compound showed lowered plasma cholinesterase but normal erythrocyte cholinesterase. A complete absence of effect would have been expected, in view of the report of Smith *et al.*(7) that administration of TOTP to albino rats by various routes in doses as high as 30 g/kg produced no response, except for pulmonary edema after intravenous injection. They concluded that the albino rat was wholly refractory.

In the rabbit, however, severe inhibition of

true cholinesterase is suggested by their report(8) of muscular tremors and other signs attributable to accumulation of acetylcholine, following peroral administration. The guinea pig showed similar signs, while the dog and monkey did not appear to absorb the compound very well from the alimentary canal.

The possibility that true cholinesterase might be inhibited, even in the rat, was suggested by some of our earlier (unpublished) experimental work. We found typical muscular signs following administration of TOTP to rats of the Long-Evans strain. We do not know why our results should differ from those of Smith and his associates. However, the results of this experiment have demonstrated an inhibition of erythrocyte cholinesterase in this strain which is even more profound than the inhibition of plasma cholinesterase.

The only other relevant investigation which we have knowledge of is a brief experiment by Aldridge(3). He injected rabbits intravenously with 6.8 mg/kg TOTP and found 81% inhibition of plasma cholinesterase after 30 minutes, but only 58% inhibition of erythrocyte cholinesterase at 90 minutes. From our results with rats, it seems likely that inhibition of erythrocyte cholinesterase would have been much greater if the blood had been tested in 12 to 24 hours.

Aldridge demonstrated in the same paper that TOTP must be metabolized before cholinesterase inhibition occurs. If different metabolites are involved in the two types of inhibition, this might account for the delayed inhibition of erythrocyte cholinesterase. However, the time difference might also be attributable to slow penetration of the red blood cell.

The results obtained with the 2 isomers of TOTP were almost as anticipated. It was not expected that either would cause any cholinesterase inhibition, and although trimetatolyl phosphate did reduce plasma activity, no other effects were noted.

Summary. 1) In contrast to previous reports, TOTP was found to be an active inhibitor of true cholinesterase in erythrocytes and brain of rats. Cholinergic signs were noted when activity in either tissue fell to less than 20% of normal. Maximum inhibition occurred 24 hours after single injection and exceeded inhibition of the plasma enzyme. Repeated sublethal doses also produced greater inhibition of true than of pseudocholinesterase. 2) On the other hand, the plasma enzyme was inhibited 24 hours after a single dose of TMTP while the cholinesterase of erythrocytes and brain was not significantly affected. TPTP failed to inhibit the enzyme of any of the 3 tissues. 3) No signs of paralysis were elicited, and no histologic lesions were discovered in the peripheral or central nervous system.

For histologic examination of tissues of the experimental rats, we are indebted to Dr. Nathan Malamud, The Langley Porter Clinic, San Francisco. We also appreciate the assistance of E. G. Rice, who performed some of the cholinesterase determinations.

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Adaptation of the Zymosan Assay of Properdin to Animal Sera. (23575)

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The reaction of zymosan with properdin of certain animal species was found to be inhibited in the presence of properdin-free human complement (RP), an essential reagent in the standard assay(1). The present report describes methods for resolving the problem, and provides examples of their applicability.

Reagent standardization and technic. The reagents (RP, R3, and zymosan) were prepared according to standard methods(1). The following technical modifications were introduced, however, to simplify procedure and allow the use of reagents standardized for other purposes; comparative tests showed that they did not affect the outcome of the assay: 1. The diluent(2) for all reagents was a triethanolamine buffered saline solution (TBS) containing divalent cation in concentrations optimal for immune hemolysis. One liter of stock aqueous solution (conc., 10 ×) contained 75.0 g NaCl, 1.0 g $MgCl_2 \cdot 6H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 28.0 ml $N(CH_2CH_2OH)_3$, and 180 ml HCl (1 N). Before use, one volume of stock solution was diluted with nine volumes of distilled water; the molarity of the diluted solution was 0.15, the pH 7.3-7.4 at 20°C. 2. The erythrocyte suspension was prepared from sheep's blood collected at least 5 days earlier in modified Alsever's solution (3). The cells were washed three times with 10-20 volumes of TBS, and the suspension adjusted with TBS to contain 5×10^8 erythrocytes/ml. The initial suspension from a given lot of blood was adjusted on the basis of hemocytometer counts. Lysates of this suspension, however, were subjected to optical density measurements as a basis for standardizing later suspensions from the same lot. Triplicate measurements were made in 12 × 75 mm cuvettes, 0.3 ml aliquants of the cells being lysed each with 1.7 ml distilled water. The optical densities† of the lysates were read at 550 mμ against a water blank, and their mean calculated. Successive suspensions were adjusted with TBS to yield lysates with mean optical densities within plus or minus 1% of that of the initial suspension. Erythrocytes so standardized were sensitized at least 10 min. before use with an equal volume of optimally-diluted(4) hemolytic antiserum. 3. The reaction volume was 0.75 ml, as in the standard assay, but zymosan and test serum were used in the same aliquot volume (0.25 ml) as RP. This was accomplished by standardizing the zymosan suspension to contain the optimal quantity(1) in 0.25 ml, and by using serial 2-fold dilutions rather than decreasing quantities of test serum. The practice had advantages in simplicity and accuracy, and avoided supplementary, volume equalizing additions of TBS. Moreover, it was possible under the conditions to express properdin titer (units/ml) directly in terms of the highest dilution of serum yielding an endpoint reaction.

Adaptation of the assay to animal sera. Method Ia. Precipitation of properdin as euglobulin and reconstitution in human R3. The euglobulin was prepared as in reference (5), the details of preparation being as follows. Measure one volume (2.0 ml) of test serum into a 40 ml centrifuge tube, and immerse in a beaker containing ice and water. While rotating tube add slowly nine volumes (18.0 ml) of 0.0027 N HCl. Stopper with a rubber stopper and let stand at least 30 minutes in the iced-water bath. Sediment the euglobulin by spinning at least 10 minutes at 2000 G in the refrigerated centrifuge (0-2°C). Decant supernate and invert tube on filter paper for draining. Redissolve euglobulin in human R3, introducing initially about 0.5 ml, and using a glass rod to facilitate resolution. Transfer euglobulin solution quantitatively to

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† The Coleman Jr. Spectrophotometer, Model 6 A, was used with adapter for 12 mm cuvettes.

a 15 ml graduated centrifuge tube, washing the 40 ml tube with successive small volumes (0.2-0.4 ml) of R3 and adding the washings to the 15 ml tube until the volume is brought to 2.0 ml. Remove any undissolved residue by spinning 10 minutes at 1000 G, and decant supernate into a 13 × 100 mm tube. Prepare serial two-fold dilutions in TBS, and add 0.25 ml to a series of 13 × 100 mm tubes containing 0.25 ml each of zymosan suspension and RP. Stopper with rubber stoppers, mix contents, and incubate 1 hour at 37°C in the water bath; shake tubes at 10 minute intervals. Centrifuge for 10 minutes at 1000 G, and decant supernates into 13 × 100 mm tubes. Test for residual C'3 using the simplified standard technic(1). *Method Ib.* Identical with method Ia except that the euglobulin was reconstituted in endpiece(6) of human complement. This alternative was suggested by Dr. L. Pillemer. Human pseudoglobulin is essentially properdin-free and contains only a residuum of C'3. Its components are more nearly complementary to euglobulin than are those of R3, but its use requires the preparation of an additional reagent. Attempts were made at first to reconstitute the euglobulins in media simpler than human R3 or endpiece. Guinea-pig, swine, canine, and chicken euglobulins dissolved in TBS reduced the hemolytic activity of human C' when incubated with the latter (1 hour at 37°C) in the absence of zymosan. Swine and canine euglobulins were anticomplementary also when reconstituted in bovine serum albumin (7%) or heat-inactivated human C'. Human euglobulin was satisfactory in all these media. *Method II. Deferred addition of RP.* Into a series of 13 × 100 mm tubes containing 0.25 ml of zymosan suspension pipette 0.25 ml of serial 2-fold dilutions of test serum. Stopper with rubber stoppers, mix contents, and incubate 1 hour at 37°C in the water bath; shake tubes at 10 minute intervals. After this primary incubation period add 0.25 ml RP to each tube, replace stoppers, mix contents and incubate an additional hour at 37°C, shaking the tubes at 10 minute intervals. Centrifuge 10 minutes at 100 G, and decant supernates into clean 13 × 100 mm tubes. Test for residual C'3 using the simplified standard technic(1).

Results. Results obtained in concurrent properdin assays using the standard (Std) and adapted methods appear in Table I; the examples are representative of the serum species. In tests of human sera, methods Ia and Ib yielded results closely comparable to those of the standard assay. Reproducibility was good, as indicated by duplicate titrations of the same sera. Method II yielded somewhat higher titers than the others, an event which was consistent with the longer period allowed for the properdin-zymosan reaction. In our experience, the standard method failed to yield clear-cut endpoints in titrations of guinea-pig, swine, canine, or chicken sera. Methods Ia, Ib, and II, however, yielded satisfactory results with guinea-pig and swine sera, and method II was applicable in the case of canine sera. None of the adaptations proved effective in titrating chicken sera, and it appeared that properdin of this species might not be demonstrable, at least by zymosan assay.‡ It should be noted in this connection that successful application of the adapted methods required some preselection of reagents as in the standard assay(1); combinations of RP and R3 found satisfactory for one species of animal serum did not necessarily yield acceptable results with another. In evaluating the adaptations, method II might be considered the procedure of choice on the basis of sensitivity and general applicability. However, it is somewhat more time consuming than the others, and it should be noted that C'3 contributed by test serum can be a source of error as in the standard assay; the influence of this component is considerably reduced in methods Ia and Ib.

Summary. The reaction of zymosan with properdin of certain animal species was found to be inhibited in the presence of properdin-free human complement (RP), an essential reagent in the standard assay. The problem was resolved by 1) precipitating the properdin as euglobulin and reconstituting for the assay in properdin-free residues (R3 or endpiece)

‡ The presence of properdin was nevertheless demonstrable in tests(7) for virucidal activity performed by Drs. J. L. Barlow, and H. Van Vunakis of the N. Y. State Health Dept.

TABLE I. Applicability of the Standard (Std) and Adapted (Ia, Ib, II) Methods in Zymosan Assay of Animal Properdin.

Serum		Assay		Hemolysis (%) in the simplified assay with serum diluted 1:						Properdin titer*
Species	Number	Date	Method	1	2	4	8	16	32	(units/ml)
Human	21457–2	7/13/57	Std		0	0	30	50+		4
			Ia		0	0	40	50+		4
			" †		0	0	50	50+		4
			Ib		0	0	45	50+		4
			II		0	0	0	50+		8
"	21457–3	8/ 7	Std		0	0	10	50		6
			Ia		0	0	40	50+		4
			Ib		0	0	15	50+		6
			" †		0	0	15	50		6
			II		0	0	0	40	50+	8
Guinea pig	52357‡	8/ 2	Std		50+	30	15	50+		
			Ia		0	0	0	50+		8
			Ib		0	0	0	50+		8
			II			0	0	10	50+	12
Swine	12557–7	8/ 6	Std		20	10	25	50+		
			Ia		0	0	10	50		6
			Ib		0	0	0	45		8
			II		10	0	0	45		8
			" †		10	0	0	50		8
Canine	73157–10	8/15	Std		50	45	35	15	20	
			Ia		50+	20	5	5	20	
			Ib		50+	50+	5	5	25	
			II			0	0	20	50	12
Chicken	80557‡	8/ 6	Std		50+ at all dilutions (1:1 through 1:32)					
			Ia							
			Ib							
			II							

* The reciprocal of the highest serum dilution causing complete inhibition of hemolysis, or if no more than 20% hemolysis was observed at the next dilution, the mean of their reciprocals.

† Duplicate assay, using a separate euglobulin preparation in the cases of methods Ia and Ib.

‡ Pooled sera of several animals.

of human complement, or 2) deferring the addition of RP until properdin-zymosan complex had formed. The methods were applied successfully in the assay of guinea-pig, swine, and canine properdin, but were ineffective in demonstrating properdin in chicken serum.

We take this occasion to thank Dr. Louis Pillemer and his associates of the Institute of Pathology, Western Reserve University, for the generous contributions in personnel training, material, and advice made since these studies were initiated. Special thanks go also to Drs. H. Van Vunakis and J. L. Barlow of the N. Y. State Health Dept. who performed the tests for virucidal activity on chicken sera, and to Mrs. Patricia Wells and Mr. Earl H. Fife, Jr., of our own department, who provided the standardized erythrocyte suspensions and reagent diluent.

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Lipid Patterns and Atherogenesis in Cholesterol-Fed Chickens. (23576)

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It is generally believed that there is a connection between blood lipids and atherosclerosis. This has led to a search for an analytical indication of tendency toward the disease that could be applied early enough in life for corrective action. The serum cholesterol concentration, the ratio of cholesterol to phospholipid(1-4), the distribution of cholesterol among the β-lipoproteins(5) and the relative amounts of cholesterol in the α- and β-lipoproteins(6-8) have all been considered with this in mind. In experimental atherosclerosis in laboratory animals the degree and character of lesions can be compared directly with analytical results. Such work in rabbits(9-11) and in chickens(12-14) has shown that correlations may vary with the experimental treatment. For example, alloxan injection in rabbits resulted in reduced aortic atheromatosis accompanied by decreased blood cholesterol/phospholipid ratio; while in chickens, administration of estrogen decreased the blood cholesterol/phospholipid ratio, but did not reduce aortic atheromatosis.

This report presents results of a study of experimental atherosclerosis produced by feeding cholesterol to a relatively large number of chickens. The degree of atheromatosis in thoracic aortas and brachiocephalic arteries has been correlated with results of analyses for total cholesterol and lipid phosphorus in plasma and for cholesterol in lipoprotein fractions.

Methods. 1) Chickens used were set out at intervals of a few weeks in 20 experiments conducted during a continuous period of 18 months starting in April 1954. They were Kerr White Leghorn cockerels, received in the laboratory at 1 or 2 days of age and housed in wire batteries. For the first 8 weeks they were fed the starter ration described in Table I. From 8 to 16 weeks of age they were fed an atherogenic diet made by replacing 7 g of corn meal in the starter ration with 2 g of USP cholesterol and 5 g of destearinated cottonseed oil. At 16 weeks of age they were fasted overnight, bled and sacrificed. 2) From the alar vein of each chicken 4 ml of whole blood were drawn and mixed with 0.7 ml of citric acid-sodium citrate-dextrose (ACD) solution (15). This yielded enough plasma for analytical procedures which follow. *Plasma fractionation.* Plasma fractionations, started the day of bleeding for all samples, were carried out on 1 ml plasma aliquots by Cohn's Method 10 as described by Lever *et al.*(16). *Cholesterol* was determined by the method of Abell, Levy, Brodie, and Kendall(17), modified by increasing the strength of alcoholic KOH solution to dissolve precipitates from plasma fractionation more easily. Plasma fractions in their centrifuge tubes, or 0.5 ml of original plasma, were dissolved in 1 ml of 33% KOH (W/W) plus 4 ml of absolute alcohol. The tubes were stoppered and placed in an incubator at 37° overnight. In the morning the mixtures were transferred to 1 oz. polyethylene bottles with 5 ml of water. Ten ml of petroleum ether were added, and the bottles were tightly closed with polyethylene screw

TABLE I. Composition of Basal Diet.

53.3 g	Yellow corn meal
30.0	Soybean meal (44% protein)
10.0	Fishmeal
2.0	Alfalfa meal
2.0	Steamed bone meal
1.5	Ground limestone
.5	Sodium chloride
.02	Manganese sulfate
.4	Choline chloride dry mix (25% choline chloride)
.2	Viadex (4000 u A and 750 u D/g)
.05	Inositol
15.0 mg	p-Aminobenzoic acid
2.0	Niacin
1.5	Calcium pantothenate
.5	Pyridoxine
.5	Riboflavin
.25	Thiamine
50.0 μg	Menadione
12.5	Biotin
5.0	Vit. B_{12}

TABLE II. Grading of Lesions in Aortas and Brachiocephalic Arteries.

Grade	Appearance
	Series 1
1/4	Single small discrete plaques
1/2-1	Large single lesions
2	Multiple plaques, involving up to 1/4 of area
3	Multiple plaques, involving 1/4-1/2 of area
4	Multiple plaques, involving >1/2 of area
5	Multiple plaques, involving virtually entire surface area
	Series 2
1/2	One or 2 min discrete plaques
1	Scattered discrete plaques
2	(a) Heavy scattered " (b) Light uniform covering
3	Heavy uniform covering
4	Extremely heavy covering, large protruding masses, stiff wall

caps and shaken vigorously for 3 minutes. After clear phase separation suitable aliquots, usually 2 ml, of the petroleum ether were evaporated to dryness in test tubes and the amounts of cholesterol were determined with modified Liebermann-Burchard reagent(17). *Recoveries.* Analyses were carried out on single samples and recoveries were checked by comparing the sum of amounts of cholesterol in the fractions with the separately determined total cholesterol. The average recovery was 99.03%. The standard deviation for individual values was ± 7.1%. *Lipid Phosphorus* was determined by King's procedure(18) on 0.1 ml portions of plasma precipitated with trichloracetic acid as recommended by Zilversmit and Davis(19). *Grading of Lesions.* The chickens were sacrificed and the thoracic aortas and brachiocephalic arteries were removed. These were examined while fresh by 2 people working independently who graded them for degree of atheromatosis and then jointly reconciled any differences in their scores. Results from 2 series of experiments are presented here. These differ from one another in the individuals who did the scoring and in the grading systems used. Grading systems are presented in Table II. In the first series, which contained 157 birds from 11 different experiments, brachiocephalic arteries and thoracic aortas were graded separately. In the second series, which contained 98 birds from 9 experiments, brachiocephalic arteries and thoracic aortas were graded as a whole.

Results. The incidence and severity of atheromatosis observed are summarized in Table III. Plaques were found in 75% of birds examined; in 83% of the first series and 62% of the second. The results of differential examination in the first series indicated a somewhat higher incidence in brachiocephalic arteries than in aortas. Six percent of the birds had brachiocephalic lesions but no aortic lesions, but none had aortic lesions without having brachiocephalic lesions. The 2 series differed in distribution of birds with various assigned grades of involvement. In the first series 42% of aortic lesions were classified as Grade 2, whereas those in the second series were more evenly distributed between Grades 1, 2, and 3.

Figs. 1-3 present the raw individual com-

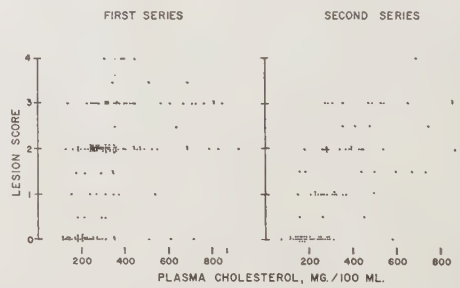

FIG. 1. Plot of plasma total cholesterol *vs* lesion score.

Table III. Distribution of Lesion scores (%)

Score	0	1/2	1	1-1/2	2	2-1/2	3	3-1/2	4
					Series 1 (157 Birds)				
Aorta	23.6	2.6	4.5	3.2	42.0	1.3	18.5	1.9	2.6
Brachiocephalics	17.2	3.2	3.8	4.5	31.2	.6	29.3	.6	9.6
					Series 2 (98 Birds)				
Aorta + Brachiocephalics	37.8	3.1	15.3	8.2	17.3	4.1	11.2	1.0	2.0

parisons of plasma cholesterol, $\frac{a}{a+\beta}$ cholesterol, and cholesterol/phospholipid ratio with lesion scores, the first series being illustrated only by the results from the thoracic aortas. Tables IV and V present correlation coefficients and regressions with their standard errors, calculated within groups, of lesion scores on all the various analytical results and ratios and their logarithms. Correlation coefficients of +1 or −1 indicate perfect association, either direct or inverse, of variables, whereas zero indicates no correlation. The listing under 100 r^2 in Table IV indicates the percentage of variation in lesion score that can be attributed to concomitant variation in corresponding lipid value.

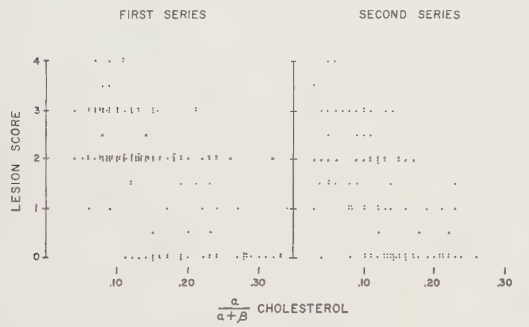

FIG. 2. Plot of plasma $\frac{a}{a+\beta}$ cholesterol *vs* lesion score.

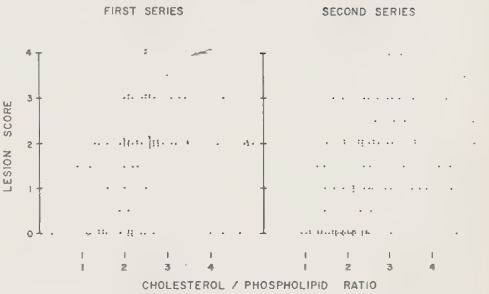

FIG. 3. Plot of plasma cholesterol/phospholipid ratio *vs* lesion score.

Discussion. In general birds with most severe atherosclerosis were also the ones with most extreme analytical values regardless of the scoring method used. The best correlations were with the logarithms of total cholesterol and β-lipoprotein cholesterol, essentially measures of the same thing in these birds. The atherosclerosis produced in chickens by

TABLE IV. Correlation Coefficients (r) of Lesion Scores on Plasma Lipid Concentrations and Ratios Expressed Arithmetically and Logarithmically.

	First series				Second series	
	Aorta score		Brachiocephalic score			
	r	(100 r^2)	r	(100 r^2)	r	(100 r^2)
Arithmetic:						
Total cholesterol, mg/100 ml	.47	(22)	.54	(29)	.55	(30)
a-Lipoprotein cholesterol, mg/100 ml	.04*	(0.1)	.09*	(0.9)	.35	(12)
β-Lipoprotein cholesterol, mg/100 ml	.50	(25)	.57	(32)	.54	(29)
$\frac{a}{a+\beta}$	−.57	(32)	−.58	(34)	−.57	(33)
Lipid phosphorus, mg/100 ml	.54	(29)	.57	(33)	.46	(21)
$\frac{\text{Cholesterol}}{\text{Phospholipid}}$	.29	(9)	.34	(11)	.61	(37)
Logarithmic:						
Total cholesterol, mg/100 ml	.57	(33)	.64	(41)	.65	(42)
a-Lipoprotein cholesterol, mg/100 ml	.07*	(0.5)	.13*	(1.7)	.35	(12)
β-Lipoprotein cholesterol, mg/100 ml	.60	(36)	.66	(44)	.64	(41)
$\frac{a}{a+\beta}$	−.49	(24)	−.51	(26)	−.29†	(8)
Lipid phosphorus, mg/100 ml	.51	(26)	.52	(27)	.54	(29)
$\frac{\text{Cholesterol}}{\text{Phospholipid}}$	.42	(17)	.41	(17)	.62	(38)

* Correlation not significant: $P > 0.1$.

† Correlation highly significant: $P = 0.005$. Correlation very highly significant in all other instances: $P < 0.001$.

TABLE V. Regression Coefficients (b), and Their Standard Errors, of Lesion Scores on Plasma Lipid Concentrations and Ratios Expressed Arithmetically and Logarithmically.

	First series		Second series
	Aorta score	Brachiocephalic score	
Arithmetic:	b ± S.E.		
Total cholesterol, mg/100 ml	.0028 ± .0005	.0034 ± .0004	.0024 ± .0004
α-Lipoprotein cholesterol, mg/100 ml	.0036 ± .0081*	.0095 ± .0084*	.0395 ± .0015†
β- " " , "	.0032 ± .0005	.0037 ± .0005	.0024 ± .0004
$\frac{\alpha}{\alpha + \beta}$	−8.6 ± 1.1	−9.2 ± 1.1	−11.1 ± 1.7
Lipid phosphorus, mg/100 ml	.43 ± .07	.46 ± .07	.21 ± .04
$\frac{\text{Cholesterol}}{\text{Phospholipid}}$	.32 ± .11†	.37 ± .11†	.89 ± .12
Logarithmic:			
Total cholesterol, mg/100 ml	3.10 ± .38	3.60 ± .37	2.95 ± .37
α-Lipoprotein cholesterol, mg/100 ml	.63 ± .73*	1.15 ± .75*	3.58 ± 1.03†
β- " " , "	2.95 ± .33	3.38 ± .32	2.72 ± .35
$\frac{\alpha}{\alpha + \beta}$	−2.57 ± .39	−2.79 ± .40	−1.01 ± .36†
Lipid phosphorus, mg/100 ml	4.26 ± .78	4.38 ± .77	4.36 ± .73
$\frac{\text{Cholesterol}}{\text{Phospholipid}}$	3.08 ± .72	3.05 ± .72	4.99 ± .68

* Regression not significant: $P > .1$.

† Regression significant: $P = .001$-$.006$. Regression very highly significant in all other instances: $P < .001$.

cholesterol feeding seems to be closely associated with resulting high plasma cholesterol concentration, most of which is in the β-lipoprotein fraction. The logarithmic expression counteracts the tendency for the data to have greater scatter at higher values.

The α-lipoprotein cholesterol was significantly related to lesion score only in the second series. There was no tendency for it to be greater in birds with little atheromatosis.

In agreement with Barr(8) it was found that the $\frac{\alpha}{\alpha + \beta}$ cholesterol ratio was the arithmetic expression most closely associated on the whole with lesion score. It offered no advantage, however, over the logarithmic expression of total cholesterol, and thus in experiments with cholesterol-fed birds the fractionation is probably not worthwhile. The cholesterol/phospholipid ratio ranked low as an indicator of lesion score in the first series, but high in the second. It is attractive to speculate that this difference may be related to differences in scoring systems, one of which considers only area of the lesions while the other tries to take into account thickness of deposit as well. However, examination of aortas from additional birds, ranked first in order of area covered and then in order of lesion thickness, did not support this conclusion. In a similar study with chickens fed ¼% cholesterol in the diet for 35 weeks Stamler and Katz(20) found that birds with aortic lesions had higher blood cholesterol than those without lesions, but found no relation with cholesterol/lipid phosphorus ratio. It would seem that degree of association of lesion score with cholesterol/phospholipid ratio varies from one experiment to another for reasons not apparent at present.

Most of the regression coefficients listed in Table V have high mathematical significance, and thus it is most improbable that the observed relationships between lipid values and lesion score are due to chance. The large number of observations contributes to this significance, and the relationships apply to the population as a whole. General conclusions can be drawn from the data. An important one is that treatments that lower the plasma cholesterol of cholesterol-fed cockerels will probably retard plaque formation also.

For individual birds, however, the plasma

lipid pattern forms an uncertain basis for estimating lesion score, just as it does for the corresponding estimation in individual human beings. If a cholesterol-fed cockerel has a plasma cholesterol concentration of 100 mg%, the chances are 1 in 2 that he will have aortic lesions. If, however, his plasma cholesterol concentration is in the neighborhood of 300 mg%, there is still 1 chance in 10 that he has no lesions at all.

The observed variance of the data can be used to calculate the reliability of the cholesterol-fed cockerel as an experimental animal. Two groups, each containing 10 birds on the regimen described here, may be expected to differ through chance from one another in average lesion score by one-half grade 3 times in 10, and by one full grade 1 time in 20. They may be expected to differ in average plasma cholesterol concentration by 10% 2 times in 3, and by 20% 1 time in 3. It is only as the difference approaches 50% that the probability of a chance occurrence decreases to 1 in 100. Thus, conclusions based upon moderate changes in lesion scores or blood cholesterol concentrations must be supported by repeated experiments.

Summary. 1) The degree of atheromatosis in the thoracic aortas and brachiocephalic arteries has been compared with plasma lipid pattern in 255 cholesterol-fed cockerels. Correlation was good with all determined variables except α-lipoprotein cholesterol concentration. It was best with logarithms of total cholesterol and β-liproprotein cholesterol concentrations, and with arithmetic $\frac{\alpha}{\alpha + \beta}$ cholesterol ratio. 2) General conclusions drawn from determined regressions are valid for population as a whole. Thus, a treatment which lowers the plasma cholesterol of cholesterol-fed cockerels may be expected to retard plaque formation also. For individual birds, however, estimation of lesion score from plasma lipid pattern is an unsatisfactory procedure. The observed variance of the data can also be used to calculate the reliability of the cholesterol-fed cockerel as an experimental animal.

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Melanocyte Stimulating Hormone Activity* of Different Pituitary Preparations.† (23577)

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Pituitary extracts seem to contain 3 distinct principles which possess MSH-activity (1). One represents the intrinsic activity present in the ACTH molecule(2-5). The other 2 principles named *alpha*-MSH and *beta*-MSH are virtually free of ACTH activity and have been isolated mainly from "posterior" (posterior and intermediate) lobe extracts. Smaller amounts of these principles have been found in the anterior lobe extracts of certain species (*e.g.*, pig, man)(6,7). Chemically the 3 MSH active principles are distinct(6,8,9). Thus *alpha*-MSH is a basic polypeptide (pK = 10-11) while *beta*-MSH is an acid polypeptide (pK = 5-6). Furthermore *beta*-MSH is precipitated with acetone at pH 6.5 while *alpha*-MSH remains in the filtrate(6,7). Oxycellulose appears to adsorb all three principles but solvent partition between dilute acid and butanol separates *alpha*-MSH in the hydrophilic fraction and *beta*-MSH and ACTH in the butanol fraction(6, 10). Recent studies have elucidated the amino acid sequence of ACTH and *beta*-MSH. A common sequence of 7 amino acids is found in both the *beta*-MSH and ACTH molecules and it is said to account for the MSH activity of these 2 polypeptides. This heptapeptide sequence consists of: Met. Glu. His. Phe. Arg. Try. Gly.(4,5). The biological activities of the various chemically distinct active principles have not been analyzed to determine whether there are any qualitative differences among the actions of the various principles. Similarly only rough approximations of the quantitative differences of their activity have been given(6).

In the present studies various pituitary extract fractions were assayed to compare quantitative and qualitative bioassay characteristics of the different active principles found in these preparations.

Materials and methods. The bioassay method has been previously described in detail(11). Briefly the method involves photoelectric reflectance measurements from the dorsal surface of intact light-adapted *Rana pipiens* before and one hour after the administration of the test substance in the dorsal lymph sac. Four-point balanced assays were performed and the results were analyzed statistically(12). This method has been found to give reproducible results over a period of a year with an average precision index (lambda) of 0.39(11). A commercial ACTH preparation (Armour Labs, lot no. L 60311) was used as the provisional "standard" of comparison in all tests. It contained 1.6 I.U./mg of ACTH. Various concentrations of the same preparation served as the "unknown" in the "blind" tests. The Posterior Pituitary Powder (2 USP units/mg) and the Pitressin Powder (92 pressor units/mg) were kindly supplied by Parke, Davis & Co. The commercial preparations of Pitressin (lot no. G-D1294C) and Pitocin (lot no. E-1482G) were utilized; they contained 0.5% chlorobutanol as preservative. The oxycellulose purified ACTH(13) was kindly supplied by Dr. E. B. Astwood. It contained approximately 80 I.U./mg of ACTH. The butanol and hydrophilic fractions were prepared in Dr. Astwood's laboratory by partition of oxycellulose ACTH between dilute acid and butanol(10). The butanol fraction contained most of the ACTH activity (100-200 I.U./mg) while the acid fraction was virtually free of such activity (less than 1 I.U./mg). All solutions were made in 0.25% acetic acid. Doses were injected into the dorsal lymph sac in a volume of 0.1 ml. Control animals received 0.1 ml of 0.25% acetic acid. In addition control animals with the

* "Melanocyte stimulating hormone" (MSH) is adopted here because it has found general acceptance among American authors, although the present writers have certain reservations regarding some connotations of this terminology.

† Supported by grant from Parke, Davis and Co., Detroit, Mich.

Pitressin and Pitocin injected groups received chlorobutanol in amounts equivalent to that injected with Pitressin and Pitocin. Following the one hour reflectance measurement, all animals were injected with a dose (32 μg/frog) of the provisional "standard" known to produce maximal response. The maximal response was measured one hour later. To facilitate comparison the results are also expressed in terms of the ED_{50} which is defined as the dose producing a photoelectrically measured response of ½ or greater of the maximal response to any dose in 50% of the animals. In arriving at the percent response of each animal the average initial and maximal reflectance measurements of each group were considered.

Results. To evaluate the accuracy of the method experimentally, 3 bioassays were made with solutions of the "standard" the concentrations of which were unknown to the persons performing the assay. The actual concentrations were disclosed after arriving at an experimental estimate from the analysis of the bioassay data. Four-point balanced assays with 5-15 animals per dose were made against known solutions of the same material. The results of these 3 tests indicated that the concentrations determined by bioassay differed from those actually present by an error ranging from -6% to +7%.

Similar 4-point balanced bioassays with 10 animals per dose were made with several pituitary fractions available. The potency ratios between the various preparations and the "standard" were calculated statistically and are given in Table I. In the same table are shown the ED_{50}s *i.e.*, the dose of each preparation which produced half-maximal or greater darkening (measured photoelectrically) in 50% of the animals. In similar tests oxytocin (Parke, Davis & Co., Pitocin) did not produce any significant melanophore response in doses up to 500 μg/frog.

Dose response lines were constructed for all the preparations tested. The slopes of these lines did not differ significantly suggesting a qualitatively similar mode of action.

Utilizing an approximately ED_{50} amount of each preparation, time-response curves were obtained by measuring light reflectance before and every 15 minutes after the injection for a period of 2-3 hours. These tests were performed in 5 animals per group. No significant differences were found among the time-response curves of the different pituitary preparations tested. All curves were similar to those previously published for the standard (11). As would be expected, boiling the "oxycellulose-ACTH" preparation in dilute NaOH for 10 minutes altered significantly its time-response curve showing a prolongation of the effect. This alkali-treated "oxycellulose-ACTH" had a potency ratio nearly five times greater than the untreated material.

TABLE I. MSH Activity of Pituitary Preparations.

Preparation	Relative potency,* stand./unknown	ED_{50}, μg/frog	Activity units, MAU/g
"Standard" (Armour ACTH)	1.00	.93	1.1×10^6
Oxycellulose purified ACTH	1.6	.58	$1.7 \times$ "
Butanol fraction of oxycellulose ACTH	.16	5.8	1.7×10^5
Hydrophylic fraction of oxycellulose ACTH	1.7	.55	1.8×10^6
Des. post. pit. powder‡	.17	5.6	1.8×10^5
Pitressin solution‡	.78	1.2†	$8.3 \times$ "
Pitressin powder‡	.58	1.6	$6.3 \times$ "

* The 95% confidence limits in each case are of the order of 30% to 200% of reported values. Thus differences smaller than about ⅓ to twice a given value cannot be considered as statistically significant.

† In terms of vol the ED_{50} of the Pitressin solution corresponds to approximately 5×10^{-3} ml of the commercial preparation.

‡ Parke, Davis & Co.

Discussion. It is apparent from the "blind" bioassays that the method may be relied upon within a ±10% accuracy. In addition the method permits the calculation of the 95% confidence limits from the internal evidence of each bioassay.

The present results suggest that the different pituitary principles act in a qualitatively similar manner on melanophores, otherwise differences in the dose-response and/or time-response curves would be expected.

Quantitatively, the butanol fraction containing mainly ACTH, and perhaps *beta*-MSH, possesses 1/10th of the MSH activity present in an equal weight of the hydrophilic fraction which contains the *alpha*-MSH. Thus the intrinsic MSH activity of ACTH can not be greater than 1/10th the activity of *alpha*-MSH. Lee and Learner(6) reported the activity of highly purified ACTH as 1/100th that of highly purified MSH.

The activity of a given preparation can only be expressed in reference to a provisional "standard." However the "standards" used by different laboratories vary greatly in their MSH activities so as to be of no value for comparative purposes among laboratories. The use of the USP Posterior Pituitary Powder as a reference standard can not be unquestionably accepted since: 1) this preparation is not standardized for its MSH activity and different lots may vary in their MSH content, 2) the stability of the MSH activity in the preparation is not known and 3) such a comparison would imply that the active fraction in the unknown has a qualitatively identical MSH activity with that present in the USP Powder.

Our results do suggest that all preparations have a qualitatively similar MSH activity which would justify the use of a common standard for all extracts. Until this point is definitely established (with chemically pure preparations), however, it would seem more appropriate to refer to the MSH potency of the various extracts in terms of their biological activity rather than by comparison with an arbitrarily chosen standard. To facilitate comparison it is proposed that a provisional unit of activity be adopted which can be defined as the amount of an active fraction producing a response (darkening) equal to or greater than ½ maximal response in 50% of the treated animals. This unit can be called melanophorotropic activity unit (MAU). Such a unit, although it is defined in terms of activity and therefore lacks in accuracy, can still be useful until a universal MSH standard is established. The definition is sufficiently rigid to permit valid semi-quantitative comparisons of the results obtained in different laboratories.

Rough calculations from the published reports indicate that the proposed unit is approximately equivalent to that used by Sulman and associates(14) nearly 10 times greater than the unit used by Lerner and associates(15), and roughly 1,000 times smaller than that of Landgrebe, Waring and associates(7). The potencies of the preparations tested are given in activity units (MAU) in Table I.

In terms of activity units the frog bioassay method is sensitive to as little as 0.01 MAU. However 10 to 40 times larger amounts are required for accurate bioassay. From the data of Lee and Lerner(6) it can be calculated that this assay method can detect 10^{-5} μg of purified *alpha*-MSH and 10^{-3} μg of pure ACTH peptide. This corresponds to approximately 10^{-4} I.U. of ACTH. Thus if the MSH activity is intrinsic to the ACTH molecule as shown recently(4,5), it would seem that the frog melanphore assay method could be utilized as a sensitive bioassay of ACTH provided that the other pituitary MSH active fractions are completely eliminated from the extracts by chemical means.

Summary. 1) Bioassays of various pituitary preparations were made to compare quantitative and qualitative characteristics of the different MSH active principles known to be present in such preparations. Results suggest that the 3 chemically distinct pituitary fractions possessing MSH activity (*i.e.*, *alpha*-MSH, *beta*-MSH, and ACTH) act on melanophores in a qualitatively similar manner. It is estimated that the intrinsic MSH activity of ACTH can not be greater than 1/10th that of *alpha*-MSH. 2) A melanophorotropic activity unit (MAU) is proposed which is defined as the amount of active principle producing ½ maximal response (or greater) in 50% of the treated animals. The approximate relationship of MAU to "units" used by other investigators is discussed. The frog bioassay is sensitive to 0.01 MAU and therefore could be used to measure as little as 10^{-3} μg of pure ACTH (corresponding to 10^{-4} I.U. of ACTH) provided this fraction can be completely separated from the other MSH active principles by previous chemical treatments, and assuming that ACTH has an in-

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Correlation of Pigmentation and Bacteriophage Sensitivity in Coagulase Positive Staphylococci. (23578)

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In the past decade several investigators have demonstrated correlations between bacteriophage type of staphylococci and antibiotic resistance, habitat or clinical disease. Sensitivity to bacteriophage seems prevalent in the coagulase positive staphylococci, although Rountree(1) and Rippon(2) have reported phages that attack coagulase negative strains. The typing phages are generally limited in range to the coagulase positive staphylococci, with 60-70% of strains typable by test dilutions of phage and the majority of the remaining strains showing some degree of sensitivity to the undiluted phages. Attempts to correlate phage sensitivity with properties other than those mentioned above have generally been unsuccessful. The present report describes the association between pigmentation and phage sensitivity first observed during phage typing of staphylococci isolated from clinical specimens.

Methods. Two series of staphylococcal cultures* freshly isolated from routine clinical specimens were streaked, as received, on plates of Staphylococcus Medium No. 110(3) and incubated at 30°C for 2 days. Typical isolated colonies were subcultured on trypticase soy agar slants for further study. The original Medium No. 110 plates were scored for pigment production and incubated an additional 2 days at room temperature (25°C). In scoring, "pigmented" included the range from deep orange to pale orange or yellow, and "white" included the various shades of white and off-white. The additional incubation period enabled us to observe a number of mixtures in which slight differences in pigmentation emerged on the third or fourth day. The 2 components of such mixtures were treated as separate strains rather than color variants if they also differed in phage pattern or other properties than pigment. Tests for coagulase production were performed by the hospital laboratory and their findings were rechecked by the slide method with diagnostic plasma (Warner-Chilcott). Mannitol fermentation and proteolytic activity on Staphylococcus Medium No.

* Obtained from Parkland Memorial Hospital and Baylor University Hospital, Dallas.

TABLE I. Phage Sensitivity of 143 Strains of Staphylococci (Selected Series).

		Total	Phage sensitive			Phage insensitive
			Typable	Nontypable	Total	
Pigmented coagulase	+	72	40	26	66	6
" "	−	7	0	0	0	7
White "	+	10	1	1	2	8
" "	−	54	0	0	0	54

110 were observed in the first series of strains and hemolysis of rabbit blood agar plates was evaluated in the second series. The phage typing methods of Blair and Carr(4) were employed throughout this study, with a series of 25 typing phages kindly supplied by Dr. John E. Blair. In the first series, all strains that were not typable with the routine test dilutions of phage were retested with undiluted typing phage. This procedure was simplified in studying the second series of strains by applying a mixture of undiluted phage at the same time that the routine test dilutions were spotted. The mixture used was composed of equal volumes of 3 different typing phages (79, 3B and 54) representing the 3 major groups. Phage sensitivity, in addition to typability, includes any visible reduction in bacterial growth produced by undiluted phage suspensions, which may or may not represent true lysis (Williams and Rippon(5)).

Results. The initial series of 143 recently isolated strains was selected to include all combinations of pigment and coagulase production together with any unusual or aberrent strains. Results are summarized in Table I. A correlation between pigmentation, coagulase production and sensitivity to bacteriophage seems apparent since 66 of 72, or 93%, of the pigmented coagulase positive strains were sensitive to phage but only two of the ten white coagulase positive strains reacted to the typing phages. Coagulase negative strains were insensitive to the phages. Gelatin liquefaction and mannitol fermentation did not correlate with phage sensitivity as did pigmentation.

Laboratory stock strains of staphylococci, cultivated and stored for two years or longer, were next studied. These included the propagating strains for the typing phages plus a series of eight strains from the departmental collection. The correlation of phage sensitivity with pigment was not as marked as with the freshly isolated strains since several of the laboratory strains, originally coagulase positive and pigmented, had become altered in either or both of these properties during maintenance and storage without loss of sensitivity to phage. No attempts were made to enhance these properties by cultural methods and they were tested in the same manner as the freshly isolated clinical strains. One propagating strain, P.S. 73, was originally isolated as a phage sensitive coagulase negative component of a mixture of coagulase positive and negative cells (Rippon(2)).

These results with the laboratory strains suggested that phage sensitivity is a relatively stable property of staphylococci. A number of hospital strains of the first series were therefore retested after they had been stored 5 to 8 months. Occasional changes in pigmentation and coagulase production were observed, but no alteration in phage sensitivity, although two typable strains were modified in their lytic pattern. The most striking change occurred in the group of 8 white coagulase positive strains that were insensitive to phage. Seven of these were coagulase negative when retested, suggesting that strains with this set of characteristics represent a markedly unstable group.

A second, and larger series of strains from clinical sources was next examined for a more critical confirmation of the observed relationship between phage sensitivity and pigmentation. This series also provided an opportunity to evaluate a simpler and more economical test for phage sensitivity. The strains in this series were unselected, except for coagulase production and all coagulase positive strains were tested as received from the hospital. Undoubtedly there is some duplication due to the prevalence of a number of "hospi-

TABLE II. Phage Sensitivity of 265 Strains of Coagulase Positive Staphylococci (Unselected Series).

	Total	Phage sensitive			Phage insensitive
		Typable	Nontypable	Total	
Pigmented	247	133	111	244	3
White	18	3	2	5	13

tal strains", but close to 40 different lytic patterns are represented among the typable strains, and the highest number of strains with the same pattern was a total of 19 sensitive to phage 44A, which was found to possess a broad host range.

The test for sensitivity to phage was simplified by using a pool of 3 undiluted typing phages, applied in a single drop at the same time as the routine test dilutions of typing phage. Hood(6) reported the successful use of pools of phage for typing, and we observed in preliminary tests that undiluted suspensions of phages 79, 3B and 54 when pooled together did not show evidence of antagonism, although they represent different major groups (I, II and III). The results, summarized in Table II, would even indicate a synergistic action of the pooled phages, in view of the high percentage of phage sensitivity observed. In this unselected series over 98% of the pigmented coagulase positive strains were phage sensitive while only 5 of 18 white strains were sensitive.

Phage sensitivity is not as highly correlated with hemolysin production as it is with pigmentation and coagulase production. Sixteen strains in this series were nonhemolytic on rabbit blood agar, including 14 in the pigmented phage sensitive group and 2 in the white phage insensitive group.

Additional studies on the mode of action of the high titer phage pool have been undertaken. One group of strains undoubtedly is sensitive to infection and lysis, either by the typing phage or by contaminants derived from lysogenic propagating strains (Rippon(2)). Another group is "inhibited" by concentrated phage which do not multiply on the cells (Williams and Rippon(5)). Studies of this "inhibition phenomenon" indicate appreciable reduction in the number of viable cells. The exact mechanism of the killing is not yet understood but probably involves neither bacteriocin-like particles nor a phage-enzyme interaction as observed by Ralston *et al.*(7).

Discussion. Several reasons may explain the failure of previous workers to find correlation between phage sensitivity and pigmentation. One factor might have been the use of laboratory stock strains of staphylococci in earlier studies, since the highest correlations were obtained with strains freshly isolated from clinical specimens. Another might have been inclusion of strains from animal sources, since the typing phages have been developed primarily for the study of strains of human origin. Levy, Rippon and Williams(8), for example, have observed that 30% of coagulase positive staphylococci from animal sources were not sensitive to typing phages, and 40% of the animal strains were white or off-white. No correlation between phage sensitivity and pigmentation was observed in their study. Another factor involves the method of testing for coagulase production. The present studies and those of Finkelstein and Sulkin† indicate that the few strains which are coagulase negative by the slide test and positive by the tube test are not sensitive to phage. In the strict sense the correlation is between pigmentation, phage sensitivity and "bound" coagulase(9).

Some taxonomic considerations are involved in this study. The work of Chapman and his associates(10) would imply a continuous gradation of staphylococci from the deeply pigmented, hemolytic, mannitol and coagulase positive pathogenic strains to the white, usually non-hemolytic, mannitol and coagulase negative strains of feeble pathogenicity. The results reported in this study indicate that sensitivity to the typing phages is characteristic of the first extreme, is absent in the other extreme and occurs with limited frequency among the intermediate forms. Sensitivity to

† *J. Bact.*, in press.

typing phages is a relatively stable characteristic. It would also seem that white, phage insensitive strains are relatively unstable and tend to lose the ability to produce coagulase.

No attempt was made to correlate phage sensitivity with potential pathogenicity, although from the relationship to pigmentation and coagulase production some degree of association does exist. Further evaluation would be required before phage sensitivity could replace the commonly accepted criteria for pathogenicity.

A phage sensitivity test, employing a drop of pooled undiluted phages would be a simple, economical and expedient procedure for routine testing of staphylococci, but at present seems limited to use as an adjunct to phage typing procedures. By itself it may serve as an index of typability since all typable strains were sensitive to the pooled phage with but one exception (lytic pattern 52/44A/81). The combination of phages in the pool was selected empirically; other combinations may serve equally well.

Summary. 1) A high correlation was observed between pigmentation, bound coagulase and sensitivity to typing phages in staphylococcal strains freshly isolated from routine clinical hospital specimens. 2) A simple method for determining phage sensitivity using a pool of 3 undiluted typing phages is described and possible applications are discussed.

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Isolation and Characterization of Two Hemoglobins Found in the Turtle, *Pseudemys scripta elegans.* (23579)

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Evidence has accumulated which indicates that a correlation exists between the taxonomic relationships of animals and the chemical and physiological properties of their hemoglobins(1). With such evidence as a background, an attempt was made to correlate paper electrophoretic migration of hemoglobins with phylogeny in Amphibia and Reptilia(2). Of the reptilian hemoglobins studied those of turtles were found to be especially interesting. Hemolysates of turtles included in families Chelydridae and Kinosternidae have one hemoglobin; those included in Emydidae, Trionychidae and Testudinidae have at least 2 hemoglobins. When the 2 hemoglobins of such turtles were fractionated by means of paper electrophoresis a trail of pigment marked the area on the paper strip between the slow and fast migrating hemoglobins. This trailing suggested that an interaction was occurring between the 2 hemoglobins. Because of these interesting properties, we decided to investigate somewhat more thoroughly the hemoglobins of one of the species in which we found 2 hemoglobins, the red eared slider turtle, *Pseudemys scripta elegans.*

Materials and methods. Turtles used in this study were captured in the New Orleans area. In captivity they were kept in con-

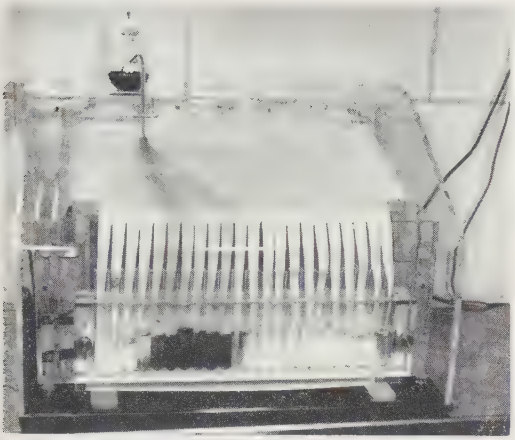

FIG. 1. Continuous flow electrophoresis. Band to right is fast Hb; band to left is slow Hb.

crete tanks located in a constant temperature room (26 to 29°C). Both a pool of water and a dry area were provided in the tanks, which were irradiated periodically with ultraviolet light. Turtles were fed rabbit and rat meat. Blood was obtained by cardiac puncture of unanesthetized turtles. The animals were turned ventral side up and a ¼ inch hole was drilled at the junction of the pectoral and abdominal laminae at the midline of the plastron. Care was taken to break through only the epidermis and bony shell so as not to damage any internal structures. A 20 gauge needle, 2½ inches long, was used to penetrate the heart. Ten to 20 ml of blood were drawn. Blood was mixed with 2 or 3 drops of a 1% heparin solution to prevent clotting. The plastral hole was closed with wax and the animal returned to the tank. Blood was processed in a refrigerated centrifuge (3 to 5°C). After centrifugation plasma was removed and erythrocytes were washed 3 times with 0.9% NaCl and hemolyzed in approximately 3 volumes of distilled water, added in a number of aliquots. The hemoglobin solution obtained was used in subsequent preparative procedures. This solution contained from 5 to 7% hemoglobin and henceforth will be designated as the hemolysate. Attempts were made to fractionate the hemolysate into its constituent hemoglobins by classical technics of salting out with ammonium sulfate or with phosphate buffers(3). These attempts were unsuccessful. Isolations were successfully carried out with a Karler-Misco* continuous-flow electrophoretic unit in either borate or barbital buffers of ionic strength 0.01 or 0.05. Separation was less successful in phosphate buffer of pH 7.4 and ionic strength 0.05. Fig. 1 demonstrates the appearance of the curtain during fractionation. The band farthest to the right (anode) is due to a hemoglobin which migrates very rapidly in a buffer of pH 8.6. For the purposes of description this hemoglobin will be designated subsequently as Fast Hb. The other component will be designated as Slow Hb. Since relatively long periods of time were required to isolate and characterize these hemoglobins precautions were observed to prevent protein denaturation. Continuous flow electrophoresis was performed in a cold room maintained at 3 to 5°C. In early preparations hemoglobins were handled as their oxygenated derivatives. Later the carbon monoxide derivative was formed before fractionation as a further precaution against denaturation or oxidation to methemoglobin. Analytical paper *electrophoresis* was carried out on an LKB unit.† A diethylbarbiturate buffer, pH 8.6, ionic strength 0.05, containing 15% glycerol by volume, was used. Runs were carried out for 17 hours at room temperature (26 to 28°C), 170 v, 4 ma. In some instances, paper strips obtained in the electrophoresis of hemolysates were stained with bromophenol blue and were scanned at 590 mμ in a Beckman DU spectrophotometer. Graphs of optical densities against distance migrated were plotted. The relative concentrations of the 2 hemoglobins of the hemolysate were estimated from the area beneath the resulting curves. Fast Hb approximated ⅓ of the total hemoglobin of the hemolysate. Moving boundary electrophoresis of dialyzed hemoglobins was carried out in a Perkin-Elmer, Model 38, Apparatus‡ operating at approximately 100 v. Specific conductance of buffered protein solutions was calculated from measurements of their specific resistance with a Leeds and Northrup bridge. Oxygen

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† Ivan Sorvall Inc., Norwalk, Conn.

‡ The Perkin-Elmer Corp., Norwalk, Conn.

TABLE I. Properties of *Pseudemys* Hemoglobins.

	Fe:O_2	Absorption maxima (mμ)					pI*	Alk. denat. (min.)†	Sed. const. (Svedbergs)
	HbO_2	Hb	HbO_2		HbCO		HbCO	HbO_2	HbCO
Fast Hb	1:1	555	576	541	568	538	5.7	4	4.6
Slow Hb	"	"	"	"	"	"	7.2	120	9.0
Hb A	"	"	"	"	"	537	6.8(3)	3	4.3 to 5.3(3)

* Isoelectric pH. † Time necessary for 50% denaturation.

capacity was measured either with a Van Slyke manometric apparatus(4) or with a Scholander syringe pipette(5). The method of Drabkin(6), slightly modified, was used to measure iron content. Sedimentation rates were determined in a Spinco Model E ultracentrifuge, accelerating at about 175,000 g (rotor temperature 23°C). To determine the alkali stability of turtle hemoglobins, an oxyhemoglobin solution of known concentration was added to a large volume of 0.1 N NaOH. The resulting solution had a pH of about 13. The rate of disappearance of the absorption band of oxyhemoglobin in the region of 540 mμ was followed in a Klett-Summerson colorimeter. *Absorption spectra* were determined with a Beckman DU spectrophotometer. Positions of absorption bands and observations of band shifts were observed with a Bausch and Lomb hand spectroscope. All absorption spectra were determined at room temperature (26 to 28°C) on solutions of the pigments in diethylbarbiturate buffer of pH 8.6, and ionic strength 0.05. Reduced hemoglobin was prepared by adding a trace of dithionite to oxyhemoglobin. Carbon monoxide hemoglobin was prepared by passing washed CO over a thin layer of hemoglobin solution. In all absorption spectra studies, solutions of human Hb A or its derivatives, dissolved in the diethylbarbiturate buffer, were used as reference standards. With the exception of studies on CO derivatives, all preparations of Hb A were purified previously by means of continuous flow electrophoresis. A simple hemolysate of human erythrocytes acted as the reference standard in CO hemoglobin studies.

Results. Preparations of Fast Hb and Slow Hb were routinely tested for homogeneity by means of paper electrophoresis. Only hemoglobins which migrated as single, sharp zones were considered to be homogeneous. Most such preparations also migrated as sharp boundaries in the moving boundary apparatus. Although electrophoretic criteria were used routinely to establish homogeneity of preparations, hemoglobins behaved as homogenous compounds in the ultracentrifuge. Constants which characterize these hemoglobins are assembled in Table I. Both turtle hemoglobins were found to be active oxygen carriers. Oxygen could be removed from both Slow HbO_2 and Fast HbO_2 either by adding traces of sodium dithionite or by evacuation. Deoxygenation of the oxyhemoglobins was accompanied by a shift in their absorption bands in the visible to the single band characteristic of reduced Hb. The absorption band of the reduced hemoglobins was replaced by the two bands of oxyhemoglobin upon reequilibration of the hemoglobins with oxygen. Absorption spectra between 460 and 680 mμ of Slow and Fast Hbs and their oxygenated derivatives were identical to the spectra of Hb A and HbO_2 A, within the resolution of the Beckman DU spectrophotometer. The specific extinction of these oxy and reduced hemoglobins, calculated at wave lengths of maximum and minimum absorption, were within 5% of those of Hb A. The agreement between the extinction coefficients was even closer for both preparations of Slow Hb and for one of the 2 preparations of Fast Hb on which absorption spectra measurements were carried out. The relatively low specific extinction of the first preparation of Fast Hb was interpreted as indicating that an oxidation of a fraction of that preparation to alkaline methemoglobin had occurred. In subsequent fractionations, the hemolysate was handled in the form of CO derivative to minimize oxidation. Positions of maxima in the visible region of

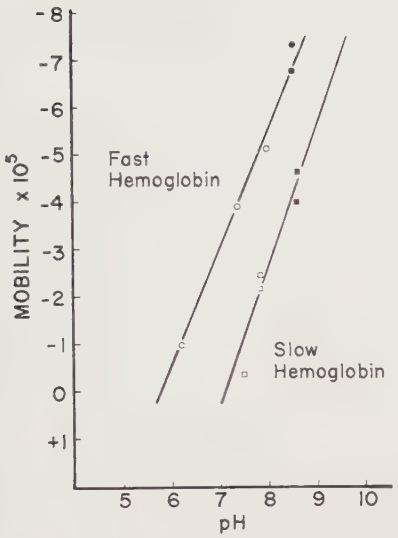

FIG. 2. Electrophoretic mobility of hemoglobins of *Pseudemys scripta elegans* in 0.1 molar ionic strength buffers. Open circles and open squares represent determinations of mobilities in phosphate buffers; closed circles and squares represent mobilities in diethylbarbiturate buffers. Protein concentrations were approximately 1%.

the electromagnetic spectrum of the CO derivatives of an hemolysate from *Pseudemys* and of Slow HbCO and Fast HbCO were identical with each other and very nearly identical with those of HbCO A.

To determine the isoelectric points of Slow HbCO and Fast HbCO, electrophoretic mobilities of 1% hemoglobin solutions were measured in buffers of varying pH and of constant ionic strength. Phosphate buffers were utilized except in the region of pH 8.6. A diethylbarbiturate buffer was used in this region. Electrophoretic mobilities obtained are plotted against pH in Fig. 2. Because of instability of the hemoglobins in acid, data are lacking in that region of the curve. Hemoglobins differ in their stability in strongly alkaline solutions. In buffers of pH 12 or above Fast HbO_2 denatures almost as rapidly as human HbO_2 A. Slow HbO_2 is very stable in alkali.

Five ultracentrifugal analyses were carried out upon samples which contained 0.2% hemoglobin. Hemolysates centrifuged both in pH 7.4 and pH 8.6 buffers resolved into 2 components, one of which sedimented approximately twice as rapidly as the other. The lighter component included about 1/3 of the total hemoglobin present in the hemolysate. Uncorrected sedimentation rates of pure samples of Slow and Fast Hbs indicated that this light component was Fast Hb and that the heavy component included Slow Hb. *In vitro* mixtures of Slow and Fast Hbs resolved into 2 components upon ultracentrifugation.

As stated previously, turtle hemoglobins appear to interact(2). In a further test of this phenomenon, 1:1 mixtures of Slow and Fast Hbs were subjected to moving boundary electrophoresis. In the ascending limb of the electrophoretic cell such mixtures resolved in the form of 2 rounded peaks; in the descending limb of the cell little or no resolution of the mixture occurred, and the pattern appeared as a single, broad, rounded peak. Although exact mobility measurements of Slow HbCO and Fast HbCO in mixtures were difficult to obtain because of this poor resolution, at pH 7.4 the mobility of the Slow component of this mixture was found to be greater than the mobility of pure Slow HbCO (-2.0×10^{-5} $cm^2/v/sec$ as compared to -0.4×10^{-5} $cm^2/v/sec$ in the ascending limb). Mobility of the Fast component was found to be less than the mobility of pure Fast HbCO (-3.6×10^{-5} $cm^2/v/sec$ as compared to -3.9×10^{-5} $cm^2/v/sec$ in the ascending limb). Measurements in diethylbarbiturate buffer indicated the same directions of mobility alterations. Moving boundary electrophoresis of a mixture of Fast HbCO and human HbCO A in a buffer of pH 8.6 indicated that interaction did not occur between these hemoglobins. Both resolved readily as sharp boundaries with no alterations in mobility. On paper, they separated as distinct spots which migrated at the same rate as adjacent samples of pure Fast Hb and pure Hb A.

Discussion. Both Fast and Slow turtle Hbs are homogeneous and active oxygen carriers. As would be expected, the absorption spectra in the visible region of these hemoglobins and of their CO and O_2 derivatives are very nearly identical to the spectra of other hemoglobins. They differ from each other and from human Hb A in isoelectric

point and in resistance to alkali. In addition, Slow Hb apparently consists of molecules of approximately twice the size of both Fast Hb and human Hb A. Svedberg and Hedenius (7) have previously reported the presence of 2 components with such differences in sedimentation constant in hemolysates of turtle blood.

Recently a pigment was found in the blood of a "desert tortoise"(8) which has the absorption spectra of a myoglobin. Such a pigment, if present in the blood of *Pseudemys,* apparently is not found in the quantities present in the tortoise blood (15 to 20% of the total circulating hemoproteins). CO derivatives of neither the unfractionated hemolysate nor of the fractions obtained by means of continuous flow electrophoresis had absorption spectra similar to the absorption spectra of CO myoglobin. Korzhuev and Kruglova reported that the oxygenated derivative of this circulatory myoglobin has absorption maxima at 582 and 543 mμ. The HbO_2 derivatives of Slow and Fast turtle Hb have absorption maxima at 576 and 541 mμ.

It is often very dangerous in comparative biochemistry to base generalizations upon observations on only a few species. An example of such a false generalization is the commonly held view that only hemoglobins of invertebrates have relatively acid isoelectric points(9). Lemberg and Legge(10) cite Svedberg and Pedersen as giving an example of a fish hemoglobin with an isoelectric point of 5.75. The presence of a hemoglobin in *Pseudemys* with an isoelectric point of 5.7 is another vertebrate for which the generalization does not hold. Data given in other work(2) also indicate that the presence in vertebrates of hemoglobins of acid isoelectric point is more common than generally realized.

Slow and Fast Hbs behave as if they interact in mixtures. The interaction was identified by the presence of a number of peculiar physicochemical properties of such mixtures. Evidence indicates that this interaction may be of a somewhat specific nature. Data obtained in physicochemical studies of turtle hemolysates are very difficult to interpret. Part of this difficulty is certainly due to the interaction which occurs between the hemoglobin components of the hemolysate.

Summary. The 2 hemoglobins of the turtle, *Pseudemys scripta elegans,* were prepared by means of a continuous flow electrophoresis. Both hemoglobins bound oxygen in a molar ratio of 1:1 with respect to iron. They have identical absorption spectra in the visible region but differ in isoelectric point, resistance to alkali, and in sedimentation constant. Physiochemical evidence is presented which indicates that these hemoglobins interact in mixtures.

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Fractionation of Serum Lactic Dehydrogenase by Salt Concentration Gradient Elution and Paper Electrophoresis.* (23580)

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The enzyme, lactic dehydrogenase (LDH), has been reported previously to be present at an elevated level in the serum of many individuals with neoplastic diseases(1,2). Although sedimentation studies have indicated the presence of only one component (3), fractionation with ammonium sulfate has revealed at least 2 components with enzymatic activity to be present. Two electrophoretic components with LDH activity have been reported in heart muscle(4,5). In the present work an attempt was made to determine whether the elevation in LDH found in the serum of some individuals with leukemia is reflected in an increase in one or more of the detectable components.

Materials and methods. Enzymatic assay. Lactic dehydrogenase activity was determined by a spectrophotometric method previously described by Hill and Levi(1). *Separation of enzyme.* Two technics have been employed in the attempts to fractionate the LDH activity: (1) *Concentration gradient elution.* The ammonium sulfate concentration gradient elution technic is a modification (6) of the method of Schwimmer(7). Serum proteins were precipitated by the addition of 9 ml of saturated ammonium sulfate solution to 1 ml of serum. The precipitated proteins were suspended in an 85% saturated ammonium sulfate solution and applied to the top of an 8 cm column of Hyflow Supercel. The suspended proteins were eluted by a steadily decreasing concentration of ammonium sulfate achieved by gradual dilution with water of an 85% saturated ammonium sulfate solution in a mixing chamber which was kept thoroughly mixed by means of a magnetic stirrer mounted beneath it. The fractions were collected in test tubes by means of an automatic fraction collector which collected 120 drops in each tube. Ammonium sulfate concentration in individual tubes was determined by nesslerization of suitable aliquots. Approximate protein concentration of all fractions was estimated by reading the optical density at 280 mμ in a Beckman DU spectrophotometer. For more precise determinations of protein concentration, the 280/260 absorption ratio was determined and protein concentration calculated according to the method of Warburg and Christian(8). (2) *Continuous flow paper electrophoresis.* Electrophoretic separation of components was carried out in the Spinco continuous flow paper electrophoresis apparatus, model CP. Prior to application sera were diluted 1:10 (for high activity serum) or 1:5 (for low activity serum) with barbiturate buffer, pH 8.6, 0.02 ionic strength. The diluted serum was then dialyzed against the same buffer overnight at 4°C. The diluted, dialyzed serum was fractionated by electrophoresis employing a current of 30 milliamperes at 450 volts. LDH activity was determined for all fractions. Upon completion of each run the paper was dried at 110° for 20 minutes, stained for 4 hours with Spinco dye B-1, washed twice with 5% acetic acid, fixed with Spinco Fixative B-2, and dried again at 110°C.

Results. Fig. 1A shows a typical column fractionation pattern of the enzyme components from the serum of a leukemic patient (high activity serum). Four peaks of enzymatic activity were obtained. In this particular experiment the eluates in tubes 8 through 39 were combined and refractionated. Again (1B) the 2 major peaks were observed but with an apparent shift of the second peak to the first. This shift has been characteristic of all results of similar experiments. Tubes 18 through 26 from the second pass of this material were combined, reprecipitated and passed through the column. The major por-

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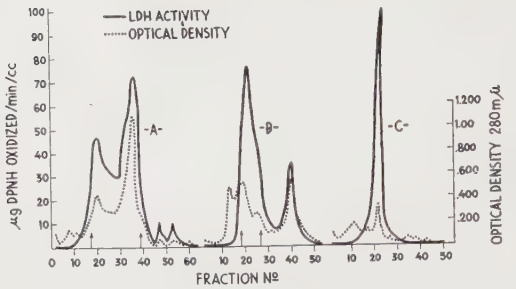

FIG. 1. Gradient elution purification of serum lactic dehydrogenase. The material used for this separation was the precipitate from 1.5 ml serum having activity equivalent to oxidation of 2.83 mg DPNH per min per ml serum. A. First pass through column; B. Second pass; C. Third pass, (see text).

tion of LDH activity now was found in 3 tubes and a protein peak was coincident with the LDH peak. The fractionation patterns obtained were essentially the same whether or not the pH of ammonium sulfate was adjusted to neutrality.

The importance of protein-protein interactions on separations of enzymes(9,10) is well known. The results in Fig. 2 show that when the highly active purified fraction is added to normal serum the activity is spread out over a larger number of tubes on a subsequent column run suggesting that such interactions may also be taking place under the conditions employed by our column procedure.

Lactic dehydrogenase has been shown to be a sulfhydryl enzyme(11,12). Since it was suspected that the shifts in peaks observed during successive fractionations might be the result of sulfhydryl interactions, fractionations were carried out on aliquots of the same serum in the absence and presence of reduced glutathione (GSH) at concentrations of 10^{-4}M. It can be seen in Fig. 3 that the separations with and without glutathione differed in that the major portion of activity was obtained in the first peak with GSH, whereas a greater spread was observed in the absence of GSH. It is of interest to note that the total activity recovered in both cases is approximately the same as determined by the areas beneath the two curves.

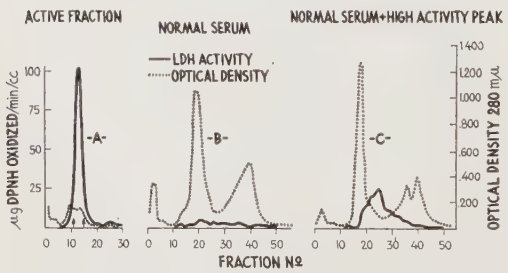

FIG. 2. Effect of serum proteins upon gradient elution fractionation. A. Active fraction from third pass through column (as in Fig. 1); B. Low activity serum (1.5 ml serum with activity equivalent to oxidation of 0.17 mg DPNH per min per ml serum); C. Active fraction (from A) and low activity serum (B) combined, (see text).

Fig. 4 shows a typical electrophoretic pattern obtained from the high activity serum of a leukemic patient. The enzymatic (LDH) activity is shown for each fraction. The same type of pattern was obtained on electrophoresis of low activity serum from apparently healthy individuals. Upon recombination of the active peaks which were rerun under the same conditions, the same electrophoretic pattern was obtained for LDH activity. This is in contrast to the results obtained with the column in which the peaks all tend to migrate to a single large peak upon repeated fractionation. The single sharp peak obtained from the column reverts to two peaks upon electrophoresis.

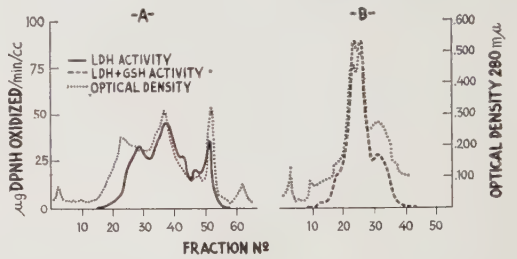

FIG. 3. Effect of glutathione upon components of LDH obtained by gradient elution fractionation of the precipitate from 1.5 ml serum having activity equivalent to oxidation of 2.83 mg DPNH per min per ml. A. No glutathione added; B. Glutathione added (1×10^{-4} M in all solutions).

Serum fractionated electrophoretically in the presence and in the absence of GSH (10^{-4}M) gave identical patterns. However, crystalline rabbit muscle LDH (Worthington Biochemical Corp.) showed a shift from a major and 2 minor components to a single major component in the presence of added GSH (10^{-4}M).

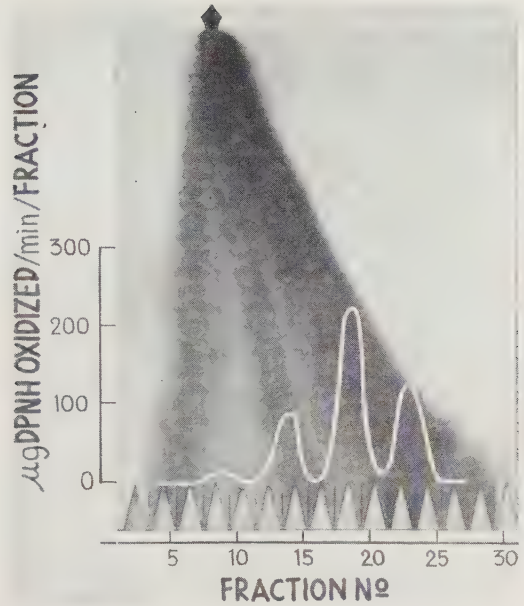

FIG. 4. Electrophoretic separation of 2 ml high activity serum (2.83 mg DPNH per min per ml). LDH activity per tube is shown superimposed on dyed paper which shows protein distribution.

Discussion. Vesell and Bearn(13) recently have reported fractionation of serum LDH into 3 distinct peaks of activity by zone electrophoresis using starch or polyvinyl as a supporting medium. These authors describe different ratios of activity in the various peaks in myocardial infarction and in leukemia. They also postulate that this may be suggestive of different forms of LDH elaborated from different sites.

All of the untreated cases of leukemia studied in our laboratories have shown a general elevation of LDH, reflected to some extent in all of the detectable constituents rather than in elevation of a single component.

At the present time it seems probable that LDH may consist of a single molecular species which can be observed as a number of components representing different states of aggregation as a result of sulfhydryl or other protein interactions. Ledoux(14) has shown in the case of ribonuclease that new forms appear during oxidation of the protein and that this can be prevented by reduced GSH. Morton and Deutsch(15) have observed similar phenomena with serum macroglobulins. We feel that perhaps we are dealing with an analogous situation with LDH.

Summary. Serum lactic dehydrogenase from leukemic and non-leukemic individuals has been separated into a number of components by ammonium sulfate concentration gradient elution and by paper electrophoresis. The effect of glutathione and of other proteins upon the fractionation patterns obtained by these two technics is described.

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Metabolism of Dihydro Streptomycin Sensitive, Resistant and Dependent *Vibrio comma.* (23581)

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Previous research of Sevag(1), Rosanoff and Sevag(2) on drug sensitive microorganisms showed that development of resistance to various drugs is accompanied by changes is metabolism. It seemed, therefore, of interest to compare certain enzymatic activities of streptomycin sensitive, resistant and dependent strains of *Vibrio comma.* A quantitative comparison of the nitratase activity, oxygen uptake and reduction of neotetrazolium was undertaken

Materials and methods. V. comma (Ogawa strain), sensitive to dihydro streptomycin was used throughout. Resistant and dependent mutants were obtained from this sensitive strain as described by Olitzki and Olitzki(3). *Casein hydrolysate egg medium* (CHEM) (4) was used throughout to obtain abundant growth of all 3 strains used. *Non-proliferating suspensions* were prepared from bacteria which had been grown at 37°C for 18 hours on solid CHEM either with or without nitrate (0.3 g/liter medium). The cells were repeatedly washed with distilled water by centrifugation until no color reaction with Gries-Ilosvay reagent was observed in the supernatant fluid. Final bacterial suspension was adjusted to the required turbidity with the aid of a Unicam Spectrophotometer. *Cell free extracts* were prepared by disintegrating the bacterial suspension in a 9KC Raytheon sonic vibrator for 20 min. and removing the cell debris by centrifugation in Sorvall centrifuge at 10,000 rpm for 10 min. *Determination of nitrate reducing activity.* The reaction mixture for determination of nitrate reducing activity contained: potassium nitrate, 5 μmoles; mannitol or another H-donor, 50 μmoles; and Sorensen phosphate buffer (pH 7.8). The mixture was kept 15 min. in water bath at 37°C and then 1 ml of either cell suspension (0.5 mg N) or cell free extract (10 mg N) was added. The volume was made up of 2.5 ml with distilled water and samples removed at intervals for nitrate determination. *Nitrite determinations* were carried out by the method of Feigl(5). Enzymatic reaction was stopped by adding 1 ml of sulphanilic acid (1% sol. in 30% glacial acetic acid). Three minutes later 1 ml of alpha-naphthylamine (0.03% sol. in 30% glacial acetic acid) was added. The volume was made to 10 ml and the color intensity was measured in the Unicam spectrophotometer at 540 mμ. A standard curve was prepared with nitrite. O_2 uptake was measured in Warburg apparatus with air as the gas phase and 15% KOH in central cup. *Neotetrazolium reduction.* According to Kun and Abood(6) the reaction mixture contained: Sorensen phosphate buffer (pH 7.8), 100 μmoles; H-donor, 30 μmoles; dihydro streptomycin, 10 μg/ml; and neo-tetrazolium, 1 mg. This mixture was kept for 15 min. in water bath at 37°C. and then the bacteria (0.5 mg N dry weight) were added. The final volume was 2 ml. The reaction was stopped when desired by addition of 5 ml of isobutanol to the reaction mixture. After shaking and standing for 24 hours at room temperature, the isobutanol was removed. This extraction was repeated with further 5 ml of isobutanol. The color intensity of the combined extracts was determined at 490 mμ, in a Unicam spectrophotometer.

Results. Nitratase activity of non-proliferating cells grown in presence and absence of nitrate. The results obtained with the various strains are summarized in Fig. 1. Similar results were obtained whether glucose or mannitol served as the H donor. The sensitive strain reduces nitrate at a much greater rate when grown in the presence of nitrate. It was surprising to find that both resistant and dependent strains failed to reduce nitrate. The complete loss of activity of the nitratase system which accompanied the change to either a resistant or a dependent strain in vibrios, was not found in the case of dihydro streptomycin resistant *Escherichia*

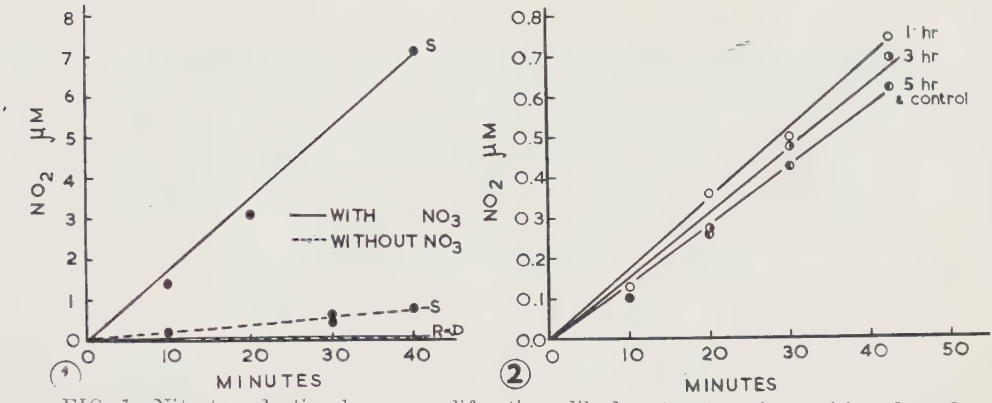

FIG. 1. Nitrate reduction by non-proliferating, dihydro streptomycin sensitive, dependent and resistant *Vibrio comma* cells. Grown in presence or absence of nitrate (0.3 g/l). Reaction mixture: H-donor, mannitol or glucose, 50 µmoles; phosphate buffer, 50 µmoles; KNO_3, 10 µmoles; total volume, 2.5 ml.

FIG. 2. Effect of preincubation with dihydro streptomycin, 10^3 µg/ml, on reduction of nitrate by sensitive *V. comma*. Reaction mixture as in Fig. 1. Bacteria grown in absence of nitrate.

coli.

Effect of preincubation with dihydro streptomycin on the nitratase activity of non-proliferating sensitive V. comma. Preincubation for 1 hour with dihydro streptomycin at concentrations of 10 to 10^4 µg/ml resulted in increased nitratase activity (Fig. 3). Preincubation with 10^5 µg/ml of dihydro streptomycin, however, had no effect on nitratase activity (Fig. 3). When preincubation of the sensitive strains with 10^3 µg/ml dihydro streptomycin was prolonged to 5 hours, only a slight reduction in observed nitratase activity, as compared to 1 hour preincubation, occurred (Fig. 2).

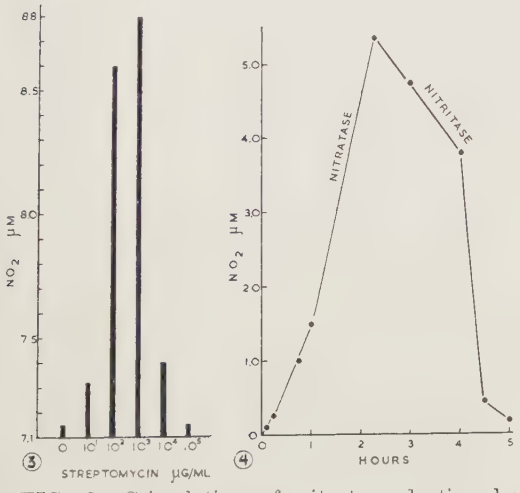

FIG. 3. Stimulation of nitrate reduction by sensitive non-proliferating *V. comma* in presence of dihydro streptomycin. Reaction mixture as in Fig. 1.; incubation 40 min.

FIG. 4. Nitrate and nitrite reduction by sensitive non-proliferating *V. comma* grown in absence of nitrate.

Nitrite reducing system in dihydro streptomycin sensitive, resistant and dependent V. comma. During determination of nitratase activity of the sensitive strain, the NO_2 content of the reaction mixture reached a maximum after 2 hours and subsequently decreased as seen in Fig. 4. The ammonia content of the reaction mixture increased as the NO_2 content fell. These observations indicate the presence in *V. comma* of a nitrite reducing enzyme system similar to those already demonstrated in other microorganisms (6-8). The resistant and dependent strains are unable to reduce nitrite.

Nitratase activity of cell free extracts of sensitive, resistant and dependent V. comma. To determine whether absence of nitratase activity was due to permeability changes in the cell membrane or whether the enzyme was affected, extracts of the 3 strains were tested for nitrate reducing activity. Cell free extracts from sensitive strains were very active but those from resistant and dependent strains were inactive. Addition of DPNH which, as previously demonstrated(8-10) is a necessary factor for the nitrate reducing system, enhanced the activity of CFE from sensitive bacteria by more than 100%, but failed to activate the CFEs from resistant or dependent

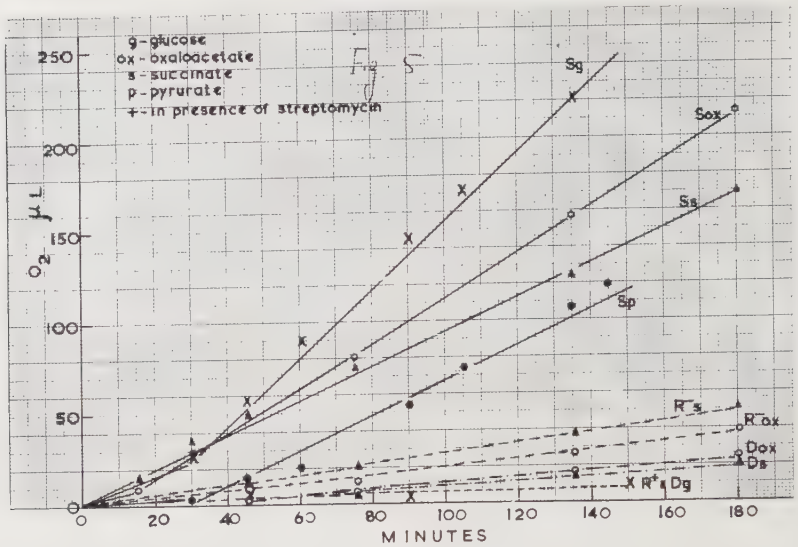

FIG. 5. Oxygen uptake by non-proliferating *V. comma* cells, sensitive—S; resistant—R; and dependent—D, to dihydro streptomycin in presence of various substrates. Substrates, 0.07 mM; dry wt cells, 0.1 mg; phosphate buffer (pH 7.8), 50 μmoles; total volume, 2 ml. All values corrected for endogenous respiration.

strains. In further experiments CFE from an active sensitive strain was added to CFE from a non-active dependent strain so that any cofactors necessary for enzymatic activity might be supplied. No additional activity was observed.

Oxygen uptake in the presence of various substrates was compared for all 3 strains. Here again striking differences were observed as seen in Fig. 5. The sensitive strains showed considerably higher rates of O_2 uptake with all substrates tested as compared to resistant and dependent strains. As for sensitive bacteria, in the presence of glucose or pyruvate, a lag period of 30 min. was observed. In the presence of oxalacetate this lag period was shorter and absent in presence of succinate.

Rate of tetrazolium reduction in presence of various substrates; effect of dihydro streptomycin. The rates of neo-tetrazolium reduction by all 3 strains were compared in the presence and absence of dihydro streptomycin. The differences are recorded in Fig. 6. The sensitive strain was very active in the presence of all substrates when tested after 45 or 105 minutes, as compared to resistant and dependent strains, which were inactive. It is of interest to note that the presence of dihydro streptomycin stimulates activity of the sensitive strain except in the presence of malate, where a retarding effect was observed. When the resistant or dependent strains were further incubated for 20 hours, slight activity could be demonstrated. It was thus demonstrated that contrary to

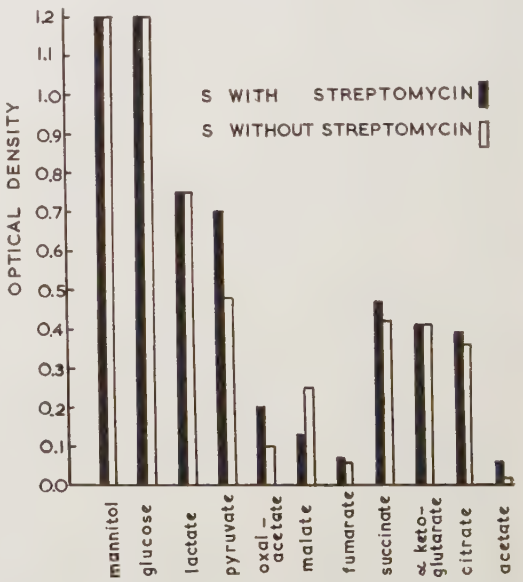

FIG. 6. Reduction of neo-tetrazolium by sensitive *V. comma* in presence of various substrates. For reaction mixture see Methods. Reaction, 105 min.

observations with sensitive bacteria, dihydro streptomycin has a retarding effect on reduction of neo-tetrazolium by resistant or dependent strains except in the case of the resistant strain in the presence of pyruvate.

Discussion. The results presented indicate that development of dependence or resistance to dihydro streptomycin in *V. comma* is accompanied by far reaching changes in the enzyme systems studied. Thus, a loss of nitratase and nitritase activity by non-proliferating *V. comma* cells, and of nitratase activity of their extracts, accompanied the development of resistance or dependence to dihydro streptomycin.

The inability of antibiotics to inhibit certain enzymatic activities of a sensitive strain, as in the case of nitratase activity and tetrazolium reduction in presence of various substrates, has also been observed with other antibiotics and microorganisms(11-14).

Differences in rate of reduction of neo-tetrazolium were observed when the 3 strains were tested in the presence of various substrates. The development of resistance or dependence was accompanied by a decrease in the neo-tetrazolium reduction rate.

In the sensitive strain the presence of the antibiotic has in fact a stimulating effect on neo-tetrazolium reduction in the presence of various substrates (except malate). Stimulation here observed is in sharp contrast to the complete inhibition (except for pyruvate) by the drug, observed in resistant and dependent strains.

Stimulation of certain enzymatic activities in a sensitive strain, in presence of the antibiotic, has also been reported by Roote and Polglase(15). Stimulation of an enzymatic reaction in an intact cell by an antibiotic, with respect to both nitrate and tetrazolium reducing activity, may result in disorganization of normal metabolic pathways.

Summary. 1) Nitrate reductase is inactive in non-proliferating dihydro streptomycin resistant and dependent *V. comma* cells and their extracts, as compared to the sensitive parent strain. Nitratase activity was found in resting cells of the sensitive strain but not in resistant and dependent mutants. Dihydro streptomycin did not inhibit but rather stimulated the nitratase activity of the sensitive strain. 2) Oxygen uptake in the presence of any of the following substrates was strongly inhibited in resistant and dependent mutant as compared to the sensitive strain: glucose, pyruvate, succinate, malate, oxaloacetate, α-ketoglutarate and fumarate. 3) Striking differences in rate of reduction of neo-tetrazolium were also observed in the presence of various substrates when the 3 strains were compared. The same pattern of high activity in the sensitive strain and practically no activity in the dependent and resistant strains was always prevalent. Here too, the presence of antibiotic resulted in stimulation of activity of the sensitive strain in the presence of most substrates tested.

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Cholesterol and Phospholipid Concentration in Hepatic Lymph and Bile During Phosphatide Induced Hypercholesteremia.* (23582)

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Hypercholesteremia induced by infusion of various phosphatides(1) does not occur unless sustained elevation of plasma phospholipid is maintained for at least several hours by continuous infusion of suitable phosphatide. This necessitates administration of a rather large amount of phosphatide (*e.g.*, 20 mg or more/hour in the rat) because of continual escape of administered phosphatide from the circulation. One of the organs responsible for this rapid withdrawal of phosphatide from plasma is the liver. This is believed to be so not only because this organ has been found(2) capable of rapidly withdrawing phospholipids from blood but also because we have observed(3) that the liverless animal invariably exhibited a far higher plasma phospholipid than the normal rat when both were infused with equivalent amounts of phosphatide.

It therefore seemed important to us to study the hepatic lymph and bile of animals receiving phosphatide in an effort to discover the possible mechanism(s) by which the liver removes excess phospholipid from the circulating blood.

Methods. A. *Effect of hepatectomy upon rate of disappearance of injected phosphatide.* A group of 14 male, adult rats (Long Evans Strain) was anesthetized and subjected to functional hepatectomy(3), immediately after which each received 90 mg of crude brain lecithin† in 3.0 ml of 5% dextrose-saline solution by immediate intravenous injection. They were bled before, immediately, and 3 hours after the injection. A group of 10 normal control rats were similarly injected with the same amount of lecithin and bled at the same intervals. The plasma samples were analyzed for phospholipid(4). B. *Effect of phosphatide infusion upon phospholipid content of hepatic and intestinal lymph.* Nine adult rats were anesthetized and after the hepatic lymph duct was cannulated and lymph began to be collected(5), each rat was injected intravenously with 3.0 ml of a 2% suspension of brain lecithin in 5% dextrose-normal-saline solution and then infused continuously at an approximate rate of 1.0 ml per hour with the same suspension of phosphatide. This infusion was continued as long as lymph continued to flow (4-12 hours). A series of 6 control rats was subjected to the identical procedures except a 5% dextrose-saline solution was substituted for the phosphatide emulsion. Plasma samples were obtained before and immediately after initial injection of the phosphatide and also at the end of the experiment. These samples together with the total lymph collections were analyzed for phospholipid and cholesterol(6). For comparative purposes, another series of 15 adult rats (starved for 36 hours prior to operation) was anesthetized, the intestinal lymph duct was cannulated(7) and the lymph collected for 10 hours. Then 8 of these 15 rats were injected and infused as described above for 10 hours with a 3% lecithin suspension in 5% dextrose-normal-saline solution. The remaining 7 rats for control purposes were infused with 5% dextrose-saline solution. Plasma samples were obtained be-

TABLE I. Rate of Disappearance of Injected Phosphatide in Liverless Rat.

No. of rats	Avg wt (g)	Plasma phospholipid (mg/100 ml) Before inj.	Immed. after inj.	3 hr after inj.
		A. Control normal rats		
10	272	110 ± 2.6*	414 ± 16.4	178 ± 7.8
		B. Hepatectomized rats		
14	274	108 ± 3.1	504 ± 18.2	421 ±12.8

* ± S.E. of mean in all cases.

* Aided by Grants from Life Insurance Medical Research Fund, Natl. Heart Inst., N.I.H., San Francisco Heart Assn. and Amer. Heart Assn.

† "Lecithin (animal), 90% pure", purchased from Nutritional Biochemicals Corp., Cleveland, O.

TABLE II. Phospholipid and Cholesterol Content of Hepatic and Intestinal Lymph during Continuous Infusion of Phospholipid.

No. of rats	Avg wt (g)	Avg amt phospholipid infused (mg)	Avg plasma phospholipid (mg/100 ml)			Avg plasma cholesterol (mg/100 ml)		Lymph			
			Before initial inj.	Immed. after initial inj.	End of infusion	Before initial inj.	End of infusion	Duration of collect. (hr)	Vol (ml)	Cholesterol (mg/100 ml)	Phospholipid (mg/100 ml)
					I. *Hepatic lymph: A. Rats infused with phosphatide*						
9	327	226 ± 11.0*	103 ± 3.2	206 ± 7.3	323 ±15.0	47 ± 2.1	117 ± 8.6	9.7 ± 1.1	2.6 ± .4	34 ± 2.4	123 ± 8.7
						B. Control rats infused with dextrose-saline					
6	320		107 ± 4.4		129 ± 3.8	51 ± 2.2	63 ± 3.6	9.8 ± 1.2	1.0 ± .2	30 ± 3.0	80 ± 7.8
					II. *Intestinal lymph: A. Rats infused with phosphatide*						
8	281	325 ± 12.0	106 ± 3.9	506 ± 7.4	1069 ± 8.2	59 ± 5.2	169 ± 8.2	10	9.9 ± .8	21 ± 1.7	88 ± 6.2
						B. Control rats infused with dextrose-saline					
7	285		104 ± 4.1		65 ± 6.2	58 ± 4.2	46 ± 3.8	10	6.3 ±1.1	26 ± 2.1	95 ± 7.4

* ± S.E. of mean in all cases.

fore and at the end of the experiment in all of the rats; in the experimental group, an additional sample was obtained immediately after the initial injection of phosphatide. The plasma samples together with the total lymph collections were analyzed usually for both phospholipid and cholesterol. C. *Effect of phosphatide infusion upon phospholipid content of hepatic bile.* A group of 10 adult rats was anesthetized, the bile duct cannulated, and bile collected for 24 hours. Five of these rats also received phosphatide as described above during the entire period of 24 hours. The remaining 5 rats received 5% dextrose-normal saline solution instead of phosphatide. Plasma samples obtained before, immediately, and 24 hours after the start of the infusion were analyzed for phospholipid and cholesterol. The total bile collection was analyzed for phospholipid.

Results. The rate of disappearance of a single injection of phosphatide was (See Table I) markedly retarded in the liverless rat. Whereas the normal animal exhibited an average decline of 57% in its plasma level of phosphatide, 3 hours after the injection, only a fall of 16% was observed in the hepatectomized rat after the same period of time.

Continuous infusion of phosphatide into rats subjected to hepatic lymph collection led to a marked elevation of plasma phosphatide (Table II) and also as a consequence of the latter elevation, the usually observed hypercholesteremia. The lymph of these rats collected during this period (Table II) as compared to the control animals exhibited little or no change in cholesterol content but a significantly increased phospholipid concentration. On the other hand, intestinal lymph obtained from a group of rats infused with phosphatide showed no higher cholesterol or phospholipid content (Table II) than the intestinal lymph of the control animals.

Similar to intestinal lymph, the bile of rats infused with phosphatide exhibited no greater content of phospholipid (Table III) than that of the control series infused with dextrose-normal saline solution.

Discussion. The results of the present experiments confirm the fact that the liver of

TABLE III. Phospholipid Content of Hepatic Bile during Continuous Infusion of Phosphatide. 5 rats/series.

Avg wt (g)	Avg amt phospholipid infused (mg)	Avg plasma phospholipid (mg/100 ml)		Avg plasma cholesterol (mg/100 ml)		Bile (24 hr)	
		Before initial inj.	Immed. after initial inj.	End of infusion†	Before initial inj.	End of infusion†	Vol (ml)	Avg phospholipid mg/100 ml mg/24 hr
A. Rats infused with phosphatide								
304	830 ± 8.0*	107 ± 2.4	305 ± 10.4	422 ± 32.0	55 ± 2.1	137 ± 18.2	14.2 ± 1.7	8.0 ± .8 1.2 ± .1
B. Control rats infused with dextrose-saline								
306		109 ± 3.0	91 ± 2.2	112 ± 2.3	54 ± 1.2	62 ± 2.2	15.6 ± .4	8.5 ± .4 1.4 ± .1

* ± S.E. of mean in all cases.

† 24 hr.

the normal rat is capable of rapidly removing large quantities of an injected phosphatide from the circulating plasma. However, it is not at all clear how phosphatide is withdrawn or what becomes of it when it is withdrawn.

Certainly the small gain in the phosphatide content of hepatic lymph suggests that little excess phosphatide accumulates in the hepatic tissue fluids despite its high concentration in the plasma and it continual escape therefrom. The failure of bile also to exhibit any increase in its phospholipid content during the massive infusion of this lipid strongly suggests that the ability of the liver to remove excess phosphatide from plasma is not due to its hepatobiliary excretory capacity. This last finding conforms with our previous observations(8) that the phospholipid rise in plasma after biliary obstruction is due primarily to a preceding rise of plasma bile acids.

It is of interest that despite the presence of hypercholesteremia in the phosphatide infused rats, no increase in the cholesterol content of either hepatic or intestinal lymph was observed. Earlier studies(9) have revealed that excess cholesterol in hepatic lymph occurs only when there is an excess in plasma of cholesterol carried in a form which is relatively freely diffusible (*e.g.,* in the rat injected with the hypercholesteremic serum obtained from a rat with biliary obstruction). Excess cholesterol does not occur in hepatic lymph when the excess plasma cholesterol is carried in a form in which it is seemingly "trapped" or inextricably bound to lipid such as occurs in the developing stages of the triglyceride induced hypercholesteremia observed in the nephrotic state(10) and after the injection of Triton(9). These latter data therefore suggest that the excess cholesterol appearing in plasma after continuous infusion of phosphatide is present in some relatively indiffusible state. Some of the excess cholesterol however does escape from the blood because the liver of the *starved* rat invariably exhibits an increase in cholesterol content after a 12 hour period of massive phosphatide infusion(3).

Summary. Rate of disappearance of intravenously injected phosphatide from plasma

was markedly retarded in the liverless rat. When hypercholesteremia was provoked in normal rats by constant infusion of phosphatide, the elevated blood cholesterol concentration was not reflected in any rise of cholesterol level in hepatic or intestinal lymph, or in bile. The elevated phospholipid level was not reflected in bile or intestinal lymph, but did cause a moderate rise in the phospholipid level of hepatic lymph.

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Effects of Dietary Fat upon Plasma Polyunsaturated Acids.* (23583)

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It has been amply demonstrated that plasma cholesterol concentration can be altered by changes in type of dietary fat(1-4). Dietary cholesterol and physiological stresses which induce hypercholesterolemia accelerate development of essential fatty acid (EFA) deficiency(5,6). It is commonly known that cholesteryl esters and phospholipids of plasma contain highly unsaturated acids. It has been postulated that polyunsaturated acids are required for normal transport of cholesterol and other lipids(7).

To test this hypothesis, a study of the effects of alterations in dietary fat upon polyunsaturated acids of plasma was desirable. The fatty acid composition of human plasma lipids was therefore determined in individuals in whom the effect of dietary fat upon plasma cholesterol was being studied. The difficulty of the analysis for polyunsaturated acids limited the number of individuals to be studied. The results presented here are from 4 individuals from Exp. I and II, and 8 individuals from Exp. III, IV, V, and VI of an investigation published earlier(8). In addition, an experiment was performed on one case of familial hypercholesterolemia in whom the effects of dietary ethyl linoleate were observed.

Exp. A. Four normal volunteers were fed experimental diets prepared under supervision of dietitian in the University Hospital in Lund. They consumed sufficient amounts of the diets to maintain their body weights constant. Blood was drawn periodically for analysis of total plasma cholesterol(9) and of plasma polyunsaturated acids(10). Two subjects were changed from their unrestricted diet to one in which 40% of calories was provided by corn oil, but in which no other fat was added. This regime was maintained for 4 weeks, then the diet was replaced for another 4 weeks by one in which butterfat provided 40% of the calories. The other 2

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TABLE I. Exp. A. Effects of Corn Oil and Butterfat upon Polyunsaturated Acids of Plasma.

Subject	Diet	Unsaturated acids as % of total lipid						
		Hexaene	Pentaene	Tetraene	Triene	Diene	Total	Tri/Tetra
G.N.	Free	.69	.79	1.78	.60	11.0	14.9	.34
	Corn oil (2 wk)	.21	.21	1.01	.51	13.3	15.2	.50
	(4 ")	.35	.42	2.72	1.31	24.0	28.8	.48
	Butterfat (2 ")	.13	.30	.97	1.07	9.2	11.7	.91
	(4 ")	.22	.46	1.32	1.13	10.2	13.3	.86
G.A.	Free	.58	.87	2.43	.79	10.2	14.9	.33
	Corn oil (2 wk)	.25	.29	1.81	.79	10.7	13.8	.44
	(4 ")	.36	1.54	1.67	1.01	15.2	19.8	.61
	Butterfat (2 ")	.31	.56	1.70	1.52	10.2	14.3	.89
S.L.	Butterfat (2 wk)	.52	.54	1.31	1.22	8.2	11.8	.91
	(4 ")	.51	1.05	2.20	1.15	9.9	14.8	.52
	Corn oil (2 ")	.33	.25	1.25	.64	15.3	17.8	.51
S.H.	Free	.57	.82	2.60	.87			.33
	Butterfat (4 wk)	.25	.64	1.83	1.41	9.5	13.6	.77
	Corn oil (2 ")	.34	.39	1.80	.98	13.2	16.7	.54
	Corn oil + chol. (2 wk)	.25	.37	1.57	1.00	10.4	13.6	.64

subjects received the 2 experimental diets in reverse order.

The analyses for polyunsaturated acids are summarized in Table I. Inasmuch as plasma is a transport vehicle in which total lipid varies, data are presented as concentrations of total lipid. As one might expect, more dienoic acid was found in plasma lipids when corn oil was fed than when butterfat was the dietary fat, for corn oil is about 50% linoleic acid. The trienoic acid concentration in plasma lipids was generally greater at times when butterfat was fed than when corn oil was fed. The tetraenoic acid contents showed no consistent pattern nor did the pentaenoic and hexaenoic acid contents. The principal and most consistent shift in the polyunsaturated acid picture aside from the reflection of dietary linoleate, was that the ratio of trienoic to tetraenoic acids varies with the kind of dietary fat. When corn oil was fed, tetraenoic acid (arachidonic acid) was the dominant non-diene polyunsaturated acid, but when butterfat was fed, the ratio of trienoic acids to tetraenoic acids increased. This is reminiscent of the increased trienoic acid contents of tissues from rats which are EFA-deficient(11).

Exp. B. In a more extensive study, effects of hydrogenated coconut oil (HCO) and corn oil upon plasma cholesterol and polyunsaturated acids were observed. In this experiment, the diets contained 40% of calories as either corn oil, hydrogenated coconut oil or a mixture of hydrogenated coconut oil and corn oil, 2:1. In another diet, 50 g corn oil daily supplemented an unrestricted diet. One subject was given 100 g lean meat daily plus 40% of calories as corn oil.

Correlation between dietary change and individual polyunsaturated acid types in plasma could not be easily seen. However, plasma contents of trienoic acid were higher when saturated fat was fed and the reverse was true for tetraenoic acids. A shift in the ratio of trienoic acids to tetraenoic acids parallels the shift in dietary fat (Table II). In this experiment the content of total polyunsaturated acids and cholesterol in plasma lipids also remained remarkably constant despite radical changes in polyunsaturated acid content of dietary fat. The cholesterol to polyunsaturated acid ratios varied from 1:1 to 3:1 with most samples near 2:1. (The ratio in cholesteryl linoleate in 1:38.) These observations may indicate that when saturated fat is a large proportion of the dietary fat transported to and from fat depots, polyunsaturated acids and cholesterol travel with it in approximately constant proportions. If transport of saturated fat is of large magnitude or of long duration, polyunsaturated acids may be released from tissue stores and cholesterol may be synthesized to travel with

TABLE II. Exp. B. Effects of Corn Oil and Hydrogenated Coconut Oil upon Plasma Polyunsaturated Acids.

Subject	Diet	Chol. Lipid	PUFA Lipid	Triene PUFA	Tetraene PUFA	Tri/Tetra
		%				
G.N.	Free	24.3	8.4	6.0	9.9	.60
	HCO (2 wk)	24.7	21.0	4.3	4.1	1.05
	HCO + CO (1 ")	24.8	15.7	5.4	8.0	.67
	" (2 ")	25.8	18.8	4.8	7.1	.34
	" (3 ")	25.0	17.4	6.8	7.8	.87
	Free + CO	24.3	13.7	8.1	9.3	.87
E.Lif.	Free	28.5	12.4	8.0	17.1	.47
	HCO (2 wk)	27.8	18.8	14.7	11.3	1.30
	CO (1 ")	25.0	14.9	3.8	8.6	.44
	" (2 ")	25.6	16.8	4.6	7.8	.59
	" + meat (1 ")	25.0	17.4	6.9	9.1	.76
	" + free	29.2	14.7	6.9	7.8	.88
J.R.	Free	24.2	11.1	9.2	12.3	.75
	HCO (2 wk)	24.0	12.9	7.0	9.3	.75
	CO (1 ")	22.0	10.1	4.1	8.0	.51
	" (2 ")	24.2	14.3	5.2	8.3	.63
	" + meat (1 ")	24.7	16.2	9.4	10.4	.90
	" + free	21.8	10.3	8.5	10.5	.81
A.L.	Free	28.1	11.7	9.6	13.2	.72
	HCO (2 wk)	27.4	14.0	19.1	11.4	1.67
	HCO + CO (1 ")	21.9	14.2	6.9	9.6	.72
	" (3 ")	22.0	13.0	7.0	8.3	.84
A.H.	Free	25.4	13.6	10.5	10.6	1.00
	HCO (2 wk)	26.6	15.2	13.0	9.9	1.31
	HCO + CO (1 ")	21.9	15.0	7.3	7.2	1.00
	" (2 ")	26.3	20.1	7.4	7.6	.97
	" (3 ")	23.6	15.7	7.7	7.9	.97
	Free (2 ")	25.2	9.4	8.9	9.4	.95
R.G.	Free	22.0	7.1	8.6	12.0	.72
	HCO (2 wk)	23.8	9.4	7.2	9.4	.77
	" (3 ")	20.5	9.6	10.2	11.2	.91
	" (4 ")	23.2	11.3	9.6	10.0	.96
	CO (1 ")	22.9	20.5	10.3	12.6	.82
	Free + CO (1 ")	24.1	8.7	10.9	10.1	1.08
E.L.	Free	22.2	10.4	8.8	10.0	.88
	HCO (2 wk)	23.9	8.1	8.1	9.5	.85
	" (3 ")	21.6	8.8	7.8	7.7	1.01
	" (4 ")	24.3	11.7	6.9	10.1	.69
	CO (1 ")	22.1	17.1	5.8	8.1	.72
	Free + CO (1 ")	24.9	11.7	5.5	7.7	.71

PUFA = polyunsaturated fatty acids; HCO = hydrogenated coconut oil; CO = corn oil.

saturated fat and to maintain homeostasis. This concept agrees with the observations that when cholesterol is fed or when animals are rendered hypercholesterolemic by hypothyroidism or diabetes, symptoms of EFA deficiency are induced more rapidly(4). Thus, essential fatty acids (or polyunsaturated fatty acids) may be regarded as necessary for normal transport of saturated fatty acids and/or cholesterol.

Exp. C. A female patient aged 44, diagnosed as familial hypercholesterolemia with xanthomatosis and angina pectoris, was studied for the effect of linoleate upon plasma cholesterol level. For some time previous to the experiment she had been on a low-fat diet, and immediately prior to the experiment she was allowed an unrestricted diet for a few days. During the first experimental period she was given a formula diet, each liter of which contained 50 g hydrogenated coconut oil and 100 g skim milk powder containing 0.8% fat. She consumed 1.5 liters of this formula daily, and her diet was supplemented with 100 g glucose, boiled rice (100 g dry weight), one portion fruit juice jelly, coffee

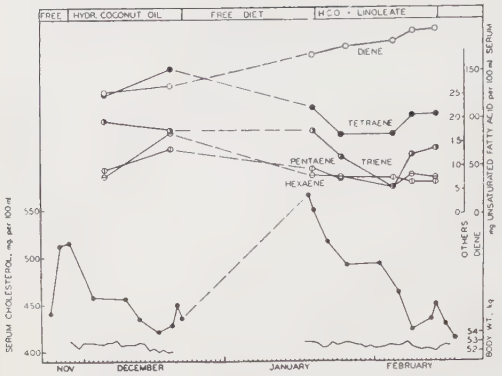

FIG. 1. Response of plasma cholesterol and polyunsaturated acids to dietary hydrogenated coconut oil and ethyl linoleate in a case of familial hypercholesterolemia.

with sugar, and one apple. Between Dec. 20, 1955 and Jan. 16, 1956, she was released and was not subject to dietary restrictions. Upon returning she was again given the formula diet, except that half of the hydrogenated coconut oil was replaced by ethyl linoleate (Jan. 19). On Jan. 30 the formula was altered to contain 25 g hydrogenated coconut oil and 20 g ethyl linoleate/liter. Vit A, B_1, C and D and iron were administered in adequate doses throughout the experiment.

The ethyl linoleate was prepared from safflower oil by repeated urea fractionation of the ethyl esters and fractional distillation to separate esters of C_{18} acids. Infrared analysis showed only a trace of *trans* unsaturation and paper chromatography revealed ethyl oleate to be the only significant impurity, approximately 5%.

The changes in plasma cholesterol and polyunsaturated acids are shown in Fig. 1. In the first experimental period, the feeding of hydrogenated coconut oil was accompanied by a decreased plasma cholesterol and by small changes in polyunsaturated acids. In the period in which ethyl linoleate was fed, plasma cholesterol concentration was reduced and dienoic acid was increased. During this time, trienoic acid and tetraenoic acids both exhibited a transitory decrease and pentaenoic and hexaenoic acids remained essentially constant. The transitory change in a polyunsaturated acid following a dietary change has been observed in other experiments, and may represent a biological readjustment. Thus, in the present experiment, the decrease in trienoic and tetraenoic acids may be a substitution of dietary linoleate for circulating polyunsaturates, followed by increased synthesis from accumulated linoleate.

Discussion. From these studies it is concluded that measurement of polyunsaturated acids in single samples of plasma does not allow one to assess the nutritive status of an individual with respect to polyunsaturated acids. This is apparent from the considerable variation between individuals and from the different degrees to which each responds to dietary change. Assessment of nutritive status is precluded because one can not determine *a priori* whether polyunsaturated acids are travelling to or from tissue stores.

This study indicates that a diet containing a high proportion of saturated fat causes a shift in polyunsaturated acids of plasma which is somewhat akin to the effect seen in tissues of animals deficient in EFA. It appears that polyunsaturated acids other than diene tend to substitute for linoleic acid when dietary fat contains little of the latter. These may be provided by mobilization of tissue polyunsaturated acids or by an increased synthesis of trienoic acid from non-essential fatty acid precursors. 5,8,11-Eicosatrienoic acid is known to increase in animals deficient in EFA(12). It is structurally related to oleic acid(13) and may therefore be a substituted metabolite produced whenever normal supplies of dietary essential polyunsaturated acids are inadequate.

Conclusion. If the increase in trienoic acids may be taken as chemical evidence of EFA deficiency, the results of this preliminary investigation suggest that prolonged ingestion of high levels of saturated fat may lead to a relative deficiency of essential fatty acids.

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Effect of Urea and Methylamine on Plasmin.* (23584)

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Plasmin, partially purified from human plasma, is unstable in neutral solutions, as demonstrated by rapid loss of proteolytic activity at 37°C. The experiments presented here show the lability to be due to self-digestion of plasmin and describe inhibition of this process by urea and methylamine. This study was undertaken after the report of the preserving effect of urea on trypsin(1), which also undergoes auto-destruction at neutral reaction. The proteolytic activity of trypsin is completely inhibited in 8 M urea, thus accounting for the ability of that concentration of urea to prevent auto-digestion of trypsin(2). The preserving and inhibiting effect is entirely reversible on sufficient dilution, indicating that trypsin does not undergo permanent change upon exposure to urea(1). With plasmin a similar situation can be shown to exist not only with urea but also with methylamine, another highly soluble simple amine.

Methods and materials. The caseinolytic assay previously described was used for determination of plasmin and plasminogen(3). The method has been modified from that originally described in that 15% rather than 10% trichloroacetic acid was added for precipitation of undigested casein. Slightly more consistent results are obtained by the use of the higher concentration of acid. *Plasmin.* The inactive precursor, plasminogen, was prepared from human plasma fraction III† by the method of Kline(4), and dried with alcohol and ether as described by Cliffton and Cannamela(5). Several lots of 400 mg were converted to plasmin as follows: The dry powder was dissolved in 30 ml distilled water with the aid of a few drops of N HCl. The volume was brought to 200 ml with 0.05 M borate-saline buffer, pH 7.4, with the formation of a finely divided precipitate. 3.0 mg commercial streptokinase‡ was added and the mixture was incubated at 25°C for 30 minutes with occasional stirring. 200 ml 2 M NaCl was added and the reaction adjusted to pH 2.0 with N HCl. As reported by Troll and Sherry, active plasmin was precipitated by this technic and streptokinase lost(6). The precipitate was separated by centrifugation for 10 minutes at 1,500 rpm and washed twice with 1 M NaCl adjusted to pH 2.0 with N HCl. The precipitate was dried by washing twice with 95% alcohol and 3 times with anhydrous ether. About

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‡ Varidase, a lyophilized mixture of streptokinase, streptodornase and other proteins with phosphate buffer, kindly furnished by Lederle Laboratories Division, Amer. Cyanamid Co., Pearl River, N. Y.

TABLE I. Loss of Plasmin Activity at pH 7.4 and 37°C.

Time (t) (min.)	Residual activity (units $\times 10^{-3}$/ml)	Fraction of initial activity (fA)	K
0	18.7	1.0	
5	12.2	.65	.0058
10	9.8	.52	.0049
15	7.5	.40	.0054
20	6.2	.33	.0054
25	5.4	.29	.0052

2 mg plasmin dissolved in 1 ml 0.0025 M HCl and 7 ml warmed buffer added at 0 time. 1 ml aliquots removed for proteolytic determinations. If diluted similarly with 0.0025 M HCl the activity of the plasmin solution was 22.8 $\times$ 10^{-3} units/ml.

200 mg of a fine white powder was recovered from each 400 mg lot; it contained about 95% of the potential protease of the starting material. Thus, a 2-fold concentration of activity was achieved. The preparation was free of streptokinase activity, and, like partially purified plasminogen, poorly soluble at neutrality but highly soluble and stable in 0.0025 M HCl. A pool of several plasmin preparations was used for all experiments described and had a specific activity of 91.2 x 10^{-3} units/mg. For each experiment a concentrated solution of the enzyme in 0.0025 M HCl was made and, at the beginning of incubation, was diluted with 5 to 10 times its volume of appropriate solution buffered at pH 7.4 with borate.

Urea and methylamine. C.P. urea and methylamine hydrochloride were dissolved in 0.05 M borate-saline buffer, pH 7.4, at 10 M and 8 M respectively and adjusted to pH 7.4 with N HCl or N NaOH. Dilution to the desired strength was made with additional borate buffer.

Results. When a solution of plasmin in 0.0025 M HCl was brought to pH 7.4 by addition of borate buffer an immediate reduction of activity was observed due to a partial reversible denaturation of protein. This was followed by a slower temperature dependent and irreversible destruction of proteolytic activity. These facts are illustrated by the experiment in Table I. The relatively slow and decreasing rate of the second reaction suggests that it may be enzymatic in nature. According to Kunitz and Northrop(7) a bimolecular reaction involving the digestion of reversibly denatured enzyme by the active enzyme should fit the formula $\frac{1}{fA} - 1 = K A_o t$ where fA is the fraction of active enzyme remaining at time t and A_o is the activity at the beginning of the experiment. Calculated values for K show good agreement and indicate that the formula fits the data.

The effect of various concentrations of urea and methylamine upon the rate of inactivation was tested. Because the urea and methylamine concentrations to be used were also inhibitory to proteolytic activity, it was necessary to allow auto-digestion to proceed in a solution with a 10-fold concentration of plasmin, remove a 0.1 ml aliquot and add it to the 1.0 ml substrate along with 0.9 ml buffer in order to dilute the urea or methylamine to a non-inhibitory level. As might be expected, concentrated solutions of plasmin digested themselves more rapidly and had higher values for K than dilute solutions. Also the rate of reaction tended to decrease after 10 to 20 minutes, a phenomenon observed for trypsin by Kunitz and Northrop (7) and explained by them as inhibition of the enzyme activity by products of digestion. For this reason, the values for K given here were determined during the first 10 minutes of incubation when inhibition was minimal.

The ability of similar concentrations of urea and methylamine to inhibit the proteolytic activity of plasmin for casein was also tested. In order to minimize the denaturing effect of these compounds on casein, they were added to the substrate just 30 seconds before plasmin was added for digestion. Despite this precaution, casein was rendered somewhat more digestible by urea, so that the values for urea given are in percent inhibition of the maximum digestion in the presence of urea.

In Fig. 1 the auto-digestion and inhibition of plasmin at various concentrations of urea is shown and an inverse relationship between inhibition and auto-digestion of plasmin demonstrated. Although at 6 M urea the value for K was extremely low, there was a slow loss of activity demonstrable during incubation over an hour or more at 37°C.

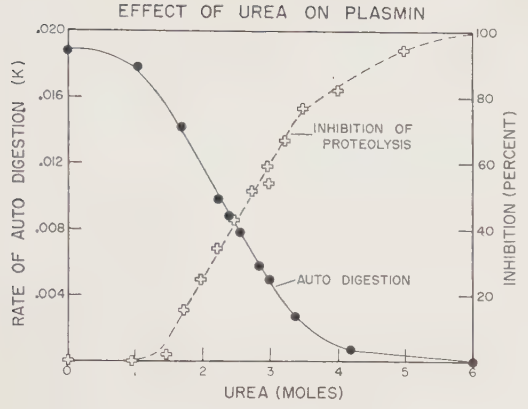

FIG. 1. Effect of urea on auto-digestion of plasmin (●). 0.1 ml 0.0025 M HCl containing 1.6 mg plasmin added to 0.6 ml buffered urea solution at 37°C. 0.1 ml aliquots removed at 1, 4, 7, and 10 min. and added to 1.0 ml 4% casein and 0.9 ml buffer for protease determination. K is an average from the 4 determinations. Inhibition of caseinolysis by urea (+). 1 ml warmed buffered urea solution added to 0.5 ml 8% buffered casein solution. 30 seconds later 0.5 ml 0.0025 M HCl containing 0.15 mg plasmin added. Activities expressed as percent of the activity in 1 M urea.

In Fig. 2 the same experiment with methylamine is shown and a similar but less perfect inverse relation is seen. Inhibition and preservation of plasmin were less complete with this compound, and at 5 M methylamine a slight increase in the rate of autolysis was found, probably due to irreversible denaturation by a high concentration. An effective preservation was seen, however, at rather low levels of methylamine.

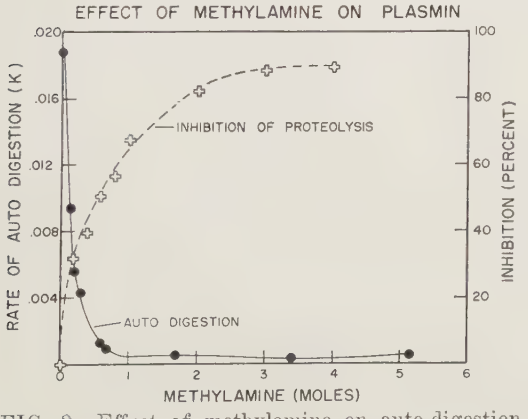

FIG. 2. Effect of methylamine on auto-digestion of plasmin (●). Inhibition of caseinolysis by methylamine (+). Activities expressed as percent of activity in buffer.

Discussion. These experiments indicate that 2 substances which inhibit proteolysis by plasmin also inhibit autolysis to about the same degree and are evidence that the lability of plasmin at neutrality is actually due to proteolytic digestion of plasmin by itself. Prevention of this process, while accomplished by urea or methylamine, can be achieved much more completely by lowering the pH. Plasmin in 0.0025 M HCl (pH 2.5) is entirely stable for many hours at 37°.

Inhibition and preservation by all 3 means (urea, methylamine, and low pH) can be correlated roughly with the ability of these conditions to make soluble this preparation of plasmin. In each case the usually poorly soluble enzyme becomes highly soluble and makes a clear solution.

In experiments with trypsin Viswanatha and Liener(1) showed that intermediate concentrations of urea actually hastened autodestruction, presumably because the denaturing effect of urea made more reversibly denatured enzyme available to destruction by the remaining native material. The lack of a similar effect with our preparation of plasmin suggests that it may already be somewhat denatured by the process of purification even though it is fully active. Its insolubility may also be due to partial denaturation.

Conclusions. 1. Preparation of partially purified and highly active human plasmin free of streptokinase is described. 2. In neutral solutions plasmin undergoes rapid auto-digestion at 37°C. 3. Autolysis of plasmin can be inhibited by concentrations of urea and methylamine which inhibit digestion of protein by plasmin.

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Effect of Fluorohydrocortisone on Gastrointestinal and Renal Excretion of Cations by the Dog.* (23585)

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The effect of adrenal cortical steroids on excretion of sodium and potassium by the kidney has been extensively studied. In contrast, there is relatively little information concerning the effect of corticosteroids on the elimination of cations via the gastrointestinal tract. The influence of the adrenal gland on the gastrointestinal transfer of sodium was first reported by Clark(1). In more recent studies, Berger(2) and Emerson(3) showed that administration of desoxycorticosterone acetate (DOCA) to rats or humans decreased fecal excretion of sodium, thereby indicating an increase in the gastrointestinal absorption of this cation. In their studies, an effect of DOCA on potassium transfer in the gut was not conclusively shown.

The present experiments performed on intact dogs demonstrated the chronic effect of large daily doses of nine-alpha-fluorohydrocortisone (FF), a synthetic analogue of hydrocortisone, on fecal and urinary excretion of sodium and potassium with particular emphasis on cation balance.

Materials and methods. Eight dogs (8 to 14 kg) from 4 different litters were used in this experiment. Of each pair from a given litter, one was the test subject and the other the control. The animals were housed individually in metabolism cages and offered a daily food ration consisting of 280 g of Gaines meal wetted with 300 ml of distilled water. By our analysis the dry meal contained 0.15 mEq of sodium and 0.22 mEq of potassium per g or 42 and 62 mEq per daily ration respectively. Since all dogs consistently ate the total ration, their daily cation intake remained uniform throughout the study. Drinking water containing less than 0.2 mEq of sodium or potassium per liter was supplied *ad libitum*. The test animals were injected intramuscularly once daily for 22 consecutive days with FF alcohol (1 mg/kg) suspended in an aqueous vehicle. Controls received the aqueous vehicle alone on a similar regimen. Measurements of water consumption and urinary and fecal outputs for a 48 hour interval were made weekly. Each dog was catheterized at the end of this period and the urine withdrawn was added to the total voided specimen. The specific gravity of the sample was determined and an aliquot saved for analysis. Stools were removed from the cages as soon as they had been passed and were stored in screw cap jars. Blood for serum was taken from the jugular vein at the mid-point of each collection period. *Sodium or potassium balance* over 48 hours was calculated by subtracting the sum total of the cation voided in urine and feces from the total quantity of that cation ingested with food. Since metabolic water and evaporative fluid losses were not accounted for, the difference between measured fluid intake and output was not considered an accurate gauge of water balance. Therefore, the conditions of the skin and mucous membranes and changes in body weight were the criteria used for estimating fluid balance. *Sodium and potassium analyses* were made on the P & I flame photometer(4) using lithium sulfate as an internal standard. Serum and urine were atomized after appropriate dilution with double distilled water. Stools were prepared for analysis as follows: each total 48 hour specimen was transferred to a tared Waring Blendor jar and its moist weight obtained by difference. The sample was blended and a 1 g aliquot removed for moisture analysis. Double distilled water was then added to the stool in sufficient quantity so that the entire sample could be reblended to a homogeneous paste. A 2 g aliquot of the paste was weighed

* We wish to thank Dr. J. Fried of Squibb Institute for the nine-alpha-fluorohydrocortisone used.

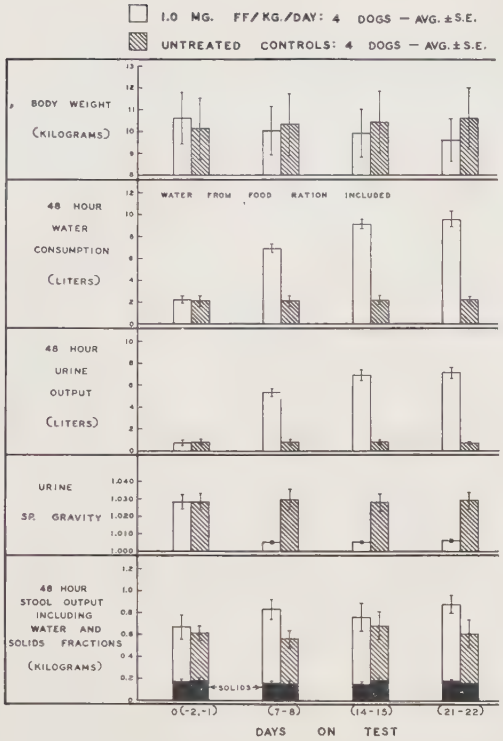

FIG. 1. Chronic effect of intramuscularly administered nine-alpha-fluorohydrocortisone (FF) on body weight, water consumption and urinary and fecal outputs of intact dogs.

in a 50 ml beaker, 10 ml of 20% chloride free nitric acid was added and the beaker covered with a short stem funnel. The sample was digested on a hot plate for about 2 hours or until the organic residue had a transparent, flocculent appearance. After cooling, the contents of the beaker were filtered quantitatively through prewashed Whatman #31 paper into a 100 ml volumetric flask containing the appropriate amount of lithium internal standard. The solution was diluted to volume and then atomized in the flame photometer. The significance of the data is evident from the graphs which include average values and their standard errors.

Results. The daily intramuscular administration of FF to dogs resulted in a striking increase in water consumption and urinary output and a decrease in urinary specific gravity (Fig. 1). Polydipsia and polyuria were well established by the end of the first week of dosage and increased during the second and third weeks of treatment. Only a small increase was noted in the excretion of fecal water and there was no change in the quantity of fecal solids. The FF treated dogs exhibited no evidence of fluid retention; rather, at the completion of the 3 weeks' dosing they showed signs of mild dehydration such as loose, tacky skin and dry mucous membranes. Moreover these dogs sustained a 9% average loss in body weight as compared to a 5% gain for controls.

The chronic polyuria produced in dogs with daily doses of FF was associated with a significant increase in the urinary excretion of sodium and a concomitant decrease in the urinary excretion of potassium (Fig. 2). Conversely, the elimination of sodium in their

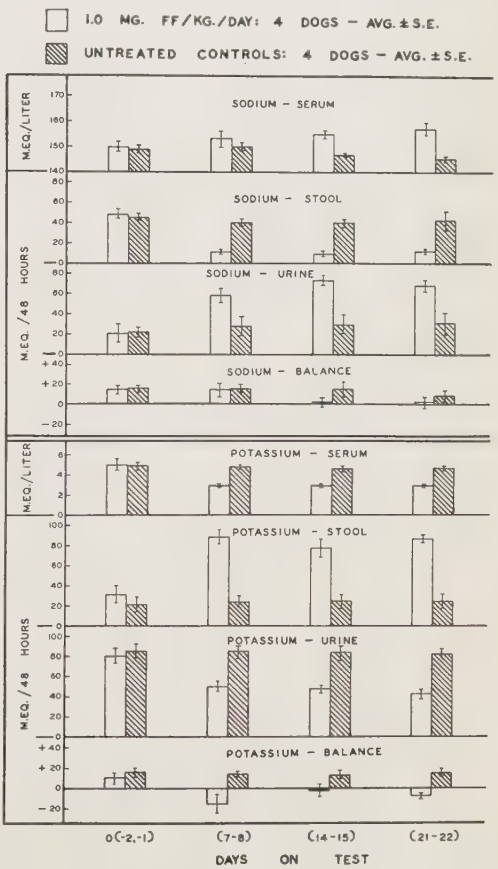

FIG. 2. Chronic effect of intramuscularly administered nine-alpha-fluorohydrocortisone (FF) on urinary and fecal excretions of sodium and potassium ions by intact dogs. Intake/48 hrs: sodium, 84 mEq; potassium, 124 mEq.

feces was sharply reduced and that of potassium was markedly increased. These changes resulted in a negative potassium balance which was reflected by an unequivocal drop in serum potassium and by slight muscular weakness. Alterations in sodium balance were not significant although directed toward the negative state. Despite the latter tendency, serum sodium levels in FF dogs showed a significant increase; this, in combination with their apparent dehydration, indicated a depletion of fluid in excess of salt.

Discussion. It is generally accepted that adrenal cortical hormones diminish the renal excretion of sodium and enhance the renal excretion of potassium. FF is reported to be extremely active in this respect(5,6). FF exerted a similar influence on sodium and potassium voidance from the gastrointestinal tract as attested to by the fecal cation changes sustained by test dogs in our experiments. Apparently, the urinary excretion of sodium and potassium in these animals was altered to offset the shift of fecal cations. Since, however, a negative potassium balance and hypokalemia with muscular weakness were produced in FF dogs, it would appear that their renal mechanism was unable to limit adequately the excretion of potassium; this effect may have been due to the profound diuresis also noted in these animals. Conversely, since no significant change was produced in sodium balance and serum sodium became elevated in the presence of dehydration, it would also appear that their renal mechanism limited the excretion of sodium, despite the copious flow of urine. Thus, although an atypical urinary pattern of cations was observed in these experiments, it is considered that the effect of FF on cation transfer in the kidney was analogous to its effect on cation transfer in the gut; with both organs functioning to abase sodium and augment potassium excretion.

Summary. The daily intramuscular administration of large doses (1 mg/kg) of nine-alpha-fluorohydrocortisone (FF) to intact dogs resulted in a chronic state of polydipsia and polyuria with gross evidence of mild dehydration. With FF treatment the fecal excretion of sodium was reduced and that of potassium enhanced but the converse was true for urinary excretion. These changes did not significantly alter sodium balance although serum sodium was elevated, but resulted in a negative potassium balance which was indicated by a drop in serum potassium and muscular weakness.

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Thymus Specificity in Lethally Irradiated Mice Treated with Rat Bone Marrow. (23586)

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The transplantation and repopulation of erythrocytic, granulocytic, and platelet precursors in lethally irradiated mice injected with heterologous (rat) bone marrow has been demonstrated by immunohematologic, cytochemical, and chromosome marker tech-

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† Operated by Union Carbide Nuclear Co. for the U. S. Atomic Energy Commission.

nics(1-5). Ford *et al.*(4) identified donor-type chromosomes in dividing cells of the thymus and lymph nodes of the protected mice and concluded that donor lymphatic tissue made up these organs; however, in the dividing cells, the actual cell type could not be identified. Merwin and Congdon(6) reported the presence of homologous donor cells in the bone marrow, spleen, blood, lymph nodes, and thymus of irradiated mice given bone marrow. Their work was based on the antigenicity of these cells in appropriate test animals. Direct evidence by another method for the repopulation of lymphatic tissues in these irradiated mice treated with bone marrow is needed.

The successful application of immunologic methods for determination of specificity of the red blood cells and platelets of lethally irradiated mice injected with rat bone marrow suggested the feasibility of similar *in vitro* methods for determining the specificity of thymus regeneration in mice given rat bone marrow.

Materials and methods. Antisera. For antirat thymus sera, thymuses from Sprague-Dawley rats were cleaned of connective tissues, weighed, "teased" apart, and made into a suspension with Tyrode's solution by flushing through 22- and 26-gauge needles. The cells were washed in 300-400 volumes of saline and adjusted to about 100 mg/ml after the last wash. Adult (101 x C_3H)F_1 mice were given 3 injections over a period of 30 days; the antigen-host relation was about 4 mg/g of body weight per injection. A modified Freund's adjuvant, consisting of antigen, Bayol F, falba, and killed tuberculosis organisms was used for the first subcutaneous injection. The second and third injections, given intraperitoneally at 10-day intervals, were cellular suspensions in Tyrode's solution. Sera were obtained 10 days after the third injection, pooled, inactivated at 56°C for ½ hour, and merthiolate added to give a final concentration of 0.1%. After standardization (see next section), aliquots of this pool were placed in small vials and frozen. Antimouse (101 x C_3H)F_1 thymus sera was obtained from Sprague-Dawley rats in a similar manner, except that the antigen-host relation was 1 mg/g of body weight per injection. Individual antisera for thymuses from 12-, 16-, and 20-week-old (101 x C_3H) F_1 mice were prepared to test the possible existence of antigenic differences for the various age groups. Cross-testing of the 3 antisera and thymuses revealed no difference in reactivity; and the antisera were pooled to form a standard reagent. A third standard antiserum consisted of a mixture of equal volumes of the antirat thymus sera and antimouse thymus sera.

Standardization of antisera. Preliminary testing of both antirat thymus sera and antimouse thymus sera showed a strong cross reaction for the respective red blood cell (RBC) type. Although the RBC contamination of thymus suspensions to be tested was negligible (less than 3%), the removal of the RBC agglutinins would provide a more specific antisera. Two absorptions with equal volumes of packed RBC (4°C, 24 hours) removed completely the hemagglutinins and diminished the reactivity for the thymus cells only slightly. By the agglutination technic to be described, tests of the antisera on suspensions of rat and mouse thymus cells mixed in various proportions showed that each antiserum could detect the specific cell type if it was present in at least 16% concentration. The technic was thus limited by its inability to detect the presence of small amounts (less than 16%) of either cell type.

Agglutination technic. Thymus cell suspensions (approximately 3-4%) were prepared in Tyrode's solution as described and a drop pipetted on a microscope slide. To this was added an equal volume of serum, and the slide was rotated gently several times to ensure proper mixing. A cover slip was placed over the mixture and microscopic examination made for agglutination of cells. Readings were taken during a 15-minute period; and the final degree of agglutination scored as −, ±, 1+, 2+, 3+, and 4+ as judged by the relative number of free cells present. For each control and experimental thymus test, five slides were prepared with the following reagents: (a) normal rat serum, (b) normal mouse serum, (c) antirat thymus serum, (d) antimouse thymus serum, and (e)

TABLE I. *In Vitro* Agglutination Tests on the Thymuses of Lethally Irradiated Mice Treated with Rat Bone Marrow.

No. of thymuses tested	Days after treatment	Serum reagent: Antirat thymus	Antimouse thymus	Antirat, antimouse thymus	Normal rat	Normal mouse
		Degree of agglutination* (mean value)				
(2)†	7	—	3+	4+	—	—
9 (4)	12	±	3+	4+	—	—
3	14	—	4+	4+	—	—
7	15	±	3+	4+	±	±
2 (2)	18	1+	3+	4+	—	—
2	20	1+	2+	4+	—	±
2 (2)	21	2±	2+	3+	±	±
2	22	2+	2+	4+	—	—
2	23	3+	1+	3+	±	±
4	25	3+	—	3+	—	±
4	28	4+	±	4+	±	±
1 (6)	29	4+	—	4+	—	—
2 (6)	30	4+	±	4+	—	—
2 (2)(2)	40	4+	±	3+	±	1+
2	45	4+	±	4+	—	—
1	55	4+	±	4+	—	±
3	56	4+	—	4+	1+	1+
1	83	4+	—	3+	—	—

* Degree of agglutination graded from — to 4+.
† Parenthetical figures indicate No. of thymuses pooled and tested as a single suspension.

antirat-antimouse thymus serum. In general, the thymus of each individual animal was tested separately, although in a few instances, the small size of the organ necessitated the pooling of several thymuses for suitable cellular suspensions (Table I). *Chromosome analysis.* In a separate set of experiments, determination of the chromosome type was made on the reticular tissues of irradiated mice treated with rat bone marrow. *Irradiation and bone marrow treatment.* Twelve-week-old male and female (101 x $C_3H)F_1$ mice were placed in a circular, perforated lucite container attached to a revolving turntable and exposed to 950 r of total-body X radiation. Radiation conditions were: 250 kv; 15 ma; ~ 160 r/min. at 60 cm; inherent filtration, 1.0 mm of Al; added filtration, 1.0 mm of Al; and hvl, 0.5 mm of Cu. Rat bone marrow (RBM) obtained from one femur and one tibia of a Sprague-Dawley rat was made into a 1-ml suspension with Tyrode's solution and injected intravenously within 4 hours after X irradiation. Mice treated with isologous (101 x $C_3H)F_1$ bone marrow (IBM) received intravenously the contents of the shaft of one femur made up in 1 ml of Tyrode's solution. At various intervals, mice were sacrificed, body and thymus weights recorded, the reticular tissues fixed in Zenker formol solution, and hematoxylin-eosin stained sections prepared. The body- and thymus-weight data were expressed in mg of thymus weight per g of body weight (T/B). This procedure was used since it showed the regeneration of the thymus relative to the recovery of the irradiated, bone marrow-treated mice. When the size of the experimental thymus permitted, one lobe was fixed for histologic study and the other made into a suspension and tested with the various reagents described previously.

Results. Immunologic tests. The results of the agglutination tests on the thymuses of 950 r-RBM mice are shown in Table I. On days 12 and 15, tests with antimouse thymus serum showed strong agglutination, but only slight reactivity was obtained with antirat thymus serum. From days 18 through 30, there was a gradual increase in the degree of agglutination when the thymuses were tested with antirat thymus serum and a corresponding decrease in reactivity with antimouse thymus serum. On the basis of these tests, the 50% level of rat and mouse thymus cells present in the test organ occurred on about the 21st day after treatment; by 30 days, all the thymus cells appeared to be of the rat

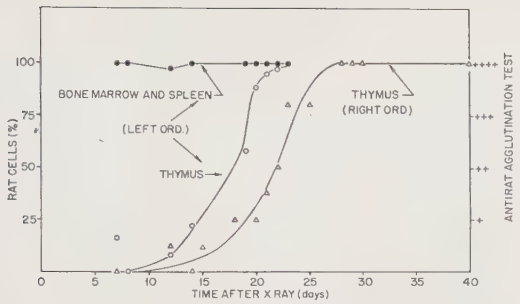

FIG. 1. Temporal relation of cytologic and immunologic identification of rat cells in bone marrow, spleen, and thymus of lethally irradiated mice treated with rat bone marrow (RBM). Cytologic, left ordinate (circles); immunologic, right ordinate (triangle).

type, as indicated by maximum agglutination. Beyond 30 days, occasional positive tests (±) were obtained with antimouse thymus serum, indicating either residual mouse thymus cells or a nonspecific type of agglutination. The positive reactions obtained with normal rat or mouse serum in several of the tests point to the latter. Although the exact cause of these reactions is not clear, their inconsistency would suggest nonspecific factors. The thymuses of 950 r-IBM mice were also tested at varying intervals after treatment. All tests showed strong agglutination with antimouse thymus serum and no reaction with antirat thymus serum (data not tabulated).

Cytological identification of tissue repopulation. Mice treated identically (950 r-RBM) to the immunologic experiment were used in a separate experiment for cytologic analysis of the proportion of mouse and rat cells in the bone marrow, spleen, lymph nodes, and thymus at increasing intervals after irradiation. By the technic of Ford and Hamerton(7), we found, as they have stated, that cells in division could be classified easily and unambiguously as either mouse or rat, on the basis of chromosomal morphology. The time course of appearance of dividing rat cells in the bone marrow, spleen, and thymus is shown in Fig. 1. Each data point for bone marrow and spleen is based on observation of 100 or more dividing cells; thymus data are from 100 cells from day 19 onward and 30 cells prior to that. On the same figure we have included, to show the temporal correlation between the effects, the appearance of rat cells in the thymus as detected immunologically (taken from Table I). It is obvious that replacement of mouse cells by rat cells occurs much sooner in the bone marrow and spleen than in the thymus. Both the bone marrow and spleen were pure rat, or almost so, by the 7th day after irradiation, whereas the thymus does not achieve this until about the 21st-22nd day. The thymus is 50% rat on about day 18, the bone marrow sometime before day 7.

Thymic weight recovery. The thymic weight pattern of lethally irradiated mice treated with RBM or IBM is shown in Fig. 2. As recorded by the average ratio of mg of thymus weight per g of body weight (T/B), a sharp reduction in size occurred within a few days after irradiation, followed in the 950 r-IBM mice by almost complete recovery within 15 days after treatment. In contrast, thymic recovery of the 950 r-RBM mice was only partial, reaching a peak value (~ 40% of normal) on the 15th-18th day, followed by a second period of relative weight loss from the 19th through the 25th days. Although the data of Fig. 2 suggest that the thymuses of these mice never completely recover, the thymus weight response varied extremely; and a few animals showed thymic recovery

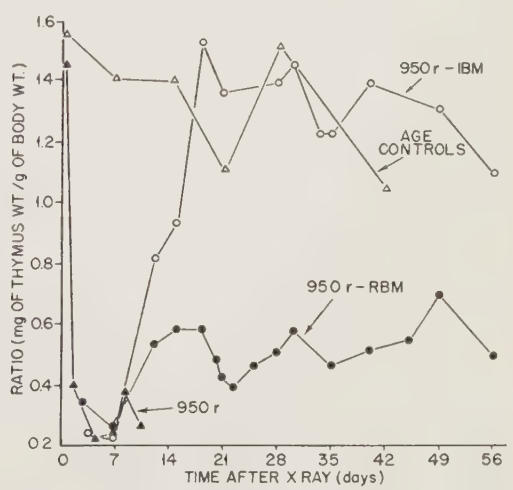

FIG. 2. Thymic recovery of lethally irradiated mice treated with rat bone marrow (RBM) or isologous bone marrow (IBM). Data represent average weights of 5-10 mice/point.

comparable to that of the IBM mice. Such variation in the T/B ratios did not occur among the 950 r-IBM mice. It was also noted that the T/B ratios of the 950 r-RBM mice that died during the experiment were well below the average value obtained with sacrificed animals.

Histologic findings. During these experiments, one or both lobes of the thymus from several sacrificed mice treated with either RBM or IBM were taken for histologic examination. For RBM mice, thymus sections were available at intervals from days 7 through 56 after irradiation. For mice treated with IBM, thymus sections were available at intervals from days 7 through 21 after irradiation.

A. *Treatment with isologous bone marrow.* On day 7 the thymus of the one animal examined showed marked atrophy (4.8 mg) with loss of cortical thymocytes. Some small, very dark-staining thymocytes were present in the medulla. In one portion of the cortex, large pale-staining thymocytes were present, suggesting early regeneration. The thymus of a mouse sacrificed on day 12 weighed 23.7 mg, and the one lobe taken for sectioning had a normal histologic architecture. On day 18, the thymus from the sacrificed mouse weighed 31 mg, and the lobe taken for sectioning was also normal histologically. The thymus on day 21 weighed 42.4 mg, and the lobe examined histologically was normal.

B. *Treatment with heterologous bone marrow.* The animal sacrificed on day 7 had a 5.6-mg thymus. One lobe was sectioned; it resembled the 7-day thymus of the animal treated with IBM. Regeneration of the cortex in some areas was more advanced, however; and several cortical cells were in mitosis. Two mice sacrificed on day 12 had thymuses weighing 17.6 and 18 mg. One lobe of each was sectioned; in both, the architecture was normal. On day 15, one lobe from each of four sacrificed animals was sectioned. These thymuses were all large, but only one was weighed (20.3 mg). Mild, irregular atrophy of the cortex was seen in one thymus. The others were normal histologically. Two animals sacrificed on day 18 had thymuses weighing 10.9 and 16.4 mg. Both showed mild to moderate irregular atrophy of the cortex in the lobe examined. On day 22, two animals sacrificed had thymuses weighing 3.1 and 7.2 mg. The smaller one showed extreme atrophy with perivascular necrosis of cells in a focal area. The larger thymus showed marked atrophy of the cortex. Many thymocytes were present, however; some of these were pycnotic, and others appeared to be normal. On days 25, 28, 40, and 56, the single animals sacrificed showed thymus weights of 9, 15, 11.9, and 17.9 mg, respectively. The 25-day thymus showed early regenerative activity in the cortex, and the medulla was packed with large thymocytes. Scattered eosinophilic leukocytes were present throughout the organ. Nearly complete regeneration of the cortex was observed in the 28-day thymus. A few eosinophils were present. Two foci of granulopoiesis in the medulla were also noted. In addition, several multinucleate giant cells were scattered through the medulla. The thymus of the 40-day mouse was normal in architecture but had slight thinning of the cortex. A few eosinophilic leukocytes were seen. In the 56-day animal, the thymus looked normal. Some eosinophilic leukocytes were present.

Discussion. The specific *in vitro* agglutination tests and the cytologic analyses indicate a repopulation of the thymus by rat-type cells in lethally irradiated mice treated with rat bone marrow. The agglutination tests showed that repopulation by rat cells was 50% complete on about the 21st day after treatment; and by 30 days, all the cells in the thymus appeared to be of the rat type. Cytological identification of dividing rat and mouse cells in the thymus showed that they were 50% rat by day 18 and almost 100% by day 21. This time difference in detection of donor cells by cytologic versus immunologic technics may be explained by the fact that cytological identification involves only the dividing cells, whereas the immunologic test is applicable to both dividing and nondividing cells. Thus, detection of remaining nondividing host cells along with the repopulating rat cells in the thymus could ac-

count for this time lag in the agglutination test.

The presence of donor-type cells in the thymuses of mice injected with homologous bone marrow (HBM) 12-14 days after treatment, as determined by their antigenicity in appropriate test animals, was reported by Merwin and Congdon(6). We verified those results in similar tests involving the experimental thymus tissue obtained from 950 r-RBM mice on the 15th and 45th days after treatment. Thus, the injections of these thymus cells into normal mice induced formation of agglutinins to rat thymus tissue, as indicated by the positive *in vitro* agglutination tests, further substantiating the heterologous origin of these cells.

The thymus weight and histology of the lethally irradiated mice treated with IBM showed a complete recovery within 21 days after exposure. In the lethally irradiated mice treated with RBM, thymus weight recovery and histologic appearance approximately paralleled that of the IBM mice for nearly 14 days after exposure. Subsequently, the thymus weight declined to a minimum by day 22. During this decline in weight, however, the immunologic test and the chromosone analysis demonstrated that the number of rat-type thymocytes was increasing relatively in the thymus, concomitant with an absolute decrease in host-type cells. A second partial recovery in thymus weight and histologic appearance took place 22-30 days after exposure. This partial recovery was, however, caused by regeneration of repopulated donor-type thymocytes.

In view of the extreme variation observed in the thymic recovery of heterologously treated mice as contrasted to the isologously treated mice, much more data are required to establish this secondary regression and recovery of the thymus as a real phenomenon. It is to be noted, however, that the time sequence in degeneration is quite similar to that observed with the secondary body weight losses occurring in 950 r-RBM mice, a phenomenon that has been attributed to the effects of a delayed *in vivo* antigen (transplanted foreign hematopoietic tissue)-antibody (recovering host's immune mechanism) reaction in the irradiated host(1,8,9). Thus the incomplete recovery of the thymus could be related indirectly to this stress mechanism within the animal. The more complete recovery of thymus tissue observed in a few animals is correlated with the failure of these mice to undergo the typical secondary loss of body weight; this emphasizes the relation of this organ's condition to the health of the irradiated host. Hirsch *et al.*(10) reported the failure of homologous and heterologous bone marrow to promote thymic regeneration after multiple sublethal total-body X irradiation, whereas regeneration was complete with isologous marrow. They suggest that different mechanisms operate in promoting thymic regeneration and survival for the different types of bone marrow treatment. Congdon and Urso(11) noted the incomplete recovery often observed with the thymus in foreign bone marrow-treated animals. They suggest that this is a result of the delayed foreign bone marrow reaction occurring in these mice.

The *in vitro* immunologic tests suggest that the temporary regeneration of thymus tissue occurring from the 7th through the 14th day after treatment is caused by a regeneration of host-type thymocytes; the chromosome analyses seem to substantiate this. However, although the latter studies indicate a greater percentage of mouse cells during this period, the low frequency of mitotic figures observed in the tissues at this time tends to negate the interpretation that this marked regeneration is caused entirely by a proliferation of host cells within the organ. The actual mechanism and factors contributing to this temporary recovery phase are still uncertain; and further experimentation is necessary to resolve this problem.

Kaplan *et al.*(12) reported that injection of parent mouse bone marrow (C_{57}BL) into X-irradiated F_1 hybrids (C_{57}BL X BALB) promoted regeneration of the irradiated thymus. Subsequent transplantation of this regenerated thymus into normal F_1 hybrids and parents resulted in a "take" and growth in the former but not in the latter hosts, suggesting that the regenerated thymus was com-

posed of host rather than donor cells. In similar experiments by Wolff and Upton (personal communication) with C_3H and (101 X $C_3H)F_1$ mice, the regenerated thymus grew when transplanted in the parent host, although the percentage of such takes was low.

In the experiments reported here, the designation of these foreign rat cells as "thymus" tissue is based primarily on their location and histologic appearance. Accepting this, one may surmise the presence of a multipotent cell in the bone marrow capable of transforming into the varying cell types and elements, as evidenced by the presence of foreign red blood cells, granulocytes, and platelets in lethally irradiated-rat-marrow-treated mice. It is also conceivable that lymphoblasts in the donor bone marrow seed the thymus gland in the irradiated host. The degree of functional ability of these foreign thymus cells as contrasted to the other transplanted foreign hematopoietic tissues is, however, open to question in view of the incomplete recovery and secondary degeneration processes often observed in this organ.

Summary. Immunologic and cytologic tests indicate that the thymuses of lethally irradiated mice protected with rat bone marrow are repopulated by rat-type cells. The agglutination tests showed this repopulation of the thymus by donor cells to be 100% complete 30 days after treatment, and the cytologic analysis of dividing cells showed 100% rat-type cells 21 days after treatment. Although the histologic architecture of these thymuses was often normal, recovery of the thymuses in terms of weight was incomplete.

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Phosphorylase and Glycogen Levels in Skeletal Muscle of Mice with Hereditary Myopathy* (23587)

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The activity of the enzyme phosphorylase, determined in biopsy samples of skeletal muscles of man with progressive muscular dystrophy, is markedly lower than normal(1). The discovery of a strain of mice with a hereditary myopathy (dystrophia muscularis), and the observation that the myopathy is in some ways similar to progressive muscular dystrophy in man(2) suggested an investigation on muscle phosphorylase in these animals. This report concerns the determination of both active and total phosphorylase and of glyogen concentration in certain skeletal muscles of these mice.

Methods. Phosphorylase activity was determined by the method of Sutherland as previously described(3) but with minor

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TABLE I. Phosphorylase Activity in Dystrophic Muscles of Mice.

	No. of mice	Dystrophic mice μg P $\pm$ S.E. P-lase a	P-lase t	Ratio a/t $\times$ 100	Control mice μg P $\pm$ S.E. P-lase a	P-lase t	Ratio a/t $\times$ 100
Thigh musc.							
A*	10	298 $\pm$ 9.2	400 $\pm$ 8.7	75 $\pm$ 3.2	424 $\pm$ 12.8	518 $\pm$ 9.8	82 $\pm$ 2.1
B†	22	179 $\pm$ 8.8	229 $\pm$ 12.6	79 $\pm$ 1.5	269 $\pm$ 7.9	348 $\pm$ 6.6	77 $\pm$ 1.3
Fore-limb musc.							
B	6	204 $\pm$ 15.2	271 $\pm$ 12.5	75 $\pm$ 3.2	306 $\pm$ 4.2	373 $\pm$ 10.7	83 $\pm$ .9
Abdominal musc.							
B	6	119 $\pm$ 3.5	165 $\pm$ 11.1	73 $\pm$ 5.9	241 $\pm$ 1.9	333 $\pm$ 5.7	72 $\pm$ .6

* A—Mice 1 mo old, enzyme activity based on 10 mg of tissue homogenate.
† B—Mice 2 mo old, enzyme activity based on 5 mg of tissue homogenate.

changes; instead of measuring the amount of glycogen synthesized in the reaction tubes, the amount of P liberated from glucose-1-phosphate was determined. This decreases the time to make an assay. Smaller quantities of muscle homogenate were employed and the incubation period was for 10 minutes at 37.5°C. Both the active *a* and the total *t* phosphorylase (requiring the addition of adenosine-5-phosphoric acid) were measured and the ratios of phosphorylase a/t were calculated. The activity of the enzyme is reported as the net increase in μg of P per unit wet weight of tissue in 10 minutes of reaction time. Glycogen concentration was determined by methods previously described(4). Dystrophic mice of both sexes were obtained from the Roscoe B. Jackson Memorial Laboratory, each with a normal littermate control of the same sex. Paired littermates were assayed simultaneously and at autopsy, were either 1 or 2 months of age. The anterior thigh muscles were usually employed but in a few instances the muscles of the upper forelimb and the abdominal wall were studied. The muscles on both sides of the mice were occasionally pooled to obtain sufficient tissue, particularly in the smaller dystrophic animals.

Results. Table I indicates that phosphorylase *a* and total phosphorylase *t* activity in the thigh muscles are both significantly lower in the mice with muscular dystrophy, ($P < .01$). However, the phosphorylase activity ratios a/t are remarkably similar with no statistically significant difference between those found in the dystrophic and normal mice. A more concentrated muscle homogenate preparation was used with the 1 month old mice and although the same general results were obtained in both age groups, no conclusion can be drawn in regards to differences in enzyme activity due to age.

The choice of the thigh muscles seemed logical at first because the dystrophic mice in walking tend to drag their hind legs and it might be expected that any differences in the enzyme activity would be readily noted. Since these mice use their forelimbs more extensively, a few determinations were made on the muscles of the upper forelimb and also on the abdominal muscles, for comparison. Table I shows a significant decrease in phosphorylase in these muscles of the dystrophic mice ($P < .01$) with no alteration in the activity ratios. The numbers of experiments are too few to comment on the relative enzyme activity among the different muscle groups except to note a rather low amount of enzyme activity in the abdominal muscles of the dystrophic mice.

Glycogen determinations were made on the thigh muscles of 11 dystrophic mice and 11 controls in the 2 month old group. The glycogen concentration expressed in mg % with the S.E. of the mean 489 $\pm$ 36 for the dystrophic mice and 352 $\pm$ 24 for the controls. The difference is statistically significant, $P < .01$.

Observations on the body weights showed that the mice with muscular dystrophy weighed on the average, 30% less than their littermate controls. This suggested a further observation on the skeleton, in particular, a measurement of the width of the tibial epi-

physeal cartilage as an index of the growth status of the mice(5). Using the method described by Geschwind and Li(5), the tibias of 7 pairs of mice were split, stained and examined. The average width of the tibial cartilage in the control was 98 $\pm$ 4.5 μ as compared to 67 $\pm$ 6.4 μ in the dystrophic mice which is significantly different, P = .02.

Discussion. Concomitant with the decrease in muscle mass in the dystrophic mouse, there is a decrease in the activity levels of muscle phosphorylase. Similar results are found in the abdominal muscles of man with progressive muscular dystrophy(1). Some enzymes are reported less active in the muscles of dystrophic mice while others are more active(6). Dreyfuss *et al.*(1) believe the changes in these enzymes are secondary to the atrophic changes in the muscles and that the decrease in enzyme activity does not necessarily represent a primary biochemical lesion. Further work is necessary to substantiate this belief.

The phosphorylase activity ratios are similar in the dystrophic and control muscles and on the basis of calculations from the data of Dreyfuss *et al.*(1), similar ratios are also found for normal and dystrophic muscle in man. The constancy of this ratio in spite of the myopathy is in contrast to the effect of denervation injury to muscle wherein there is a decrease in the a/t ratio as well as in the activity levels of phosphorylase(7). Phosphorylase activity ratios are readily decreased by induced contraction of muscle hence care must be taken in handling the muscles prior to analysis(3).

The higher concentration of glycogen seen in the thigh muscles of the dystrophic mice as compared to that in the controls is surprising and difficult to explain although the relative inactivity of these muscles in locomotion might account for it. However, these results conform to previous findings in that there is no correlation between the amount of glycogen present and phosphorylase activity in a muscle(3).

The casual observation on the difference in width of the tibial epiphyseal cartilages in the mice suggests that some hormonal disturbance may be involved which may or may not be independent of the alterations in the associated muscles. Further investigations on the skeleton as well as muscle seems appropriate since both systems are involved in the characteristic weight loss in the dystrophic mice.

Conclusions. In skeletal muscles of mice with hereditary *dystrophia muscularis,* (1) the "active" and "total" phosphorylase activities were significantly less than normal, (2) the phosphorylase activity ratios were normal, (3) the glycogen concentration was higher than normal. The body weights and the width of the tibial epiphyseal cartilages in the dystrophic mice were less than normal.

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Release and Restoration of Pituitary Lactogen in Response to Nursing Stimuli in Lactating Rats.* (23588)

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Lactogenic hormone is thought to be reflexly released from the hypophysis in response to the sucking stimulus, regular application of which seems to be essential for the maintenance of milk secretion(1). In light of this concept, the influence of 3 hours nursing upon lactogen discharge has been studied in the rat(2), rabbit and guinea pig(3). We thought it desirable to reinvestigate this phenomenon in the lactating rat in an effort to obtain more complete data which could serve as a basis for future work concerning neurohumoral mechanisms involved in lactogen release. In the present study, we have shown release of lactogen following nursing in the lactating rat occurs much more rapidly than previously determined, but subsequent restoration is a much slower process. We have also shown differences exist in pituitary lactogen levels which are reflected in total lactational performance in two strains of rats studied.

Materials and methods. Forty primiparous lactating rats of the Wistar-Missouri strain and 60 of the Sprague-Dawley-Rolfsmeyer strain were housed in individual cages and fed Purina Lab Chow and water *at libitum*. Shortly after birth each litter was reduced to 6 young and when 14 days old was isolated from their mother for 10 hours. Maternal and litter weights were recorded at this time. Fifteen rats of each strain then were immediately killed. The remaining animals were reunited with their litters and allowed to nurse for exactly 30 minutes, then killed either immediately, 2½ or 9½ hours after nursing. After death of the mothers, their pituitaries were removed, weighed individually, and frozen until assayed for lactogenic hormone. Five glands were pooled for each assay. These were crushed in a small agate mortar, suspended in a measured amount of distilled water and assayed in adult common pigeons by the Reece-Turner intradermal method(2).

Results. Although the Wistar lactating rats were much smaller, their litters at the 14th day postpartum were almost as heavy as those of Sprague-Dawley mothers (Table I). However, there was 15% more pituitary gland/100 g in the Wistar animals and, after 10 hours of isolation without nursing, assayed 30% more hormone than pituitaries of Sprague-Dawley mothers (3.50 and 2.44 Reece-Turner units/100 g, respectively) (Fig. 1.) Following 30 minutes nursing the pituitary lactogen level dropped 33% (1.16 units/100 g) in Wistar and 32% (0.78 unit/100 g) in Sprague-Dawley rats. About one-half the prenursing level was restored in both strains within 2½ hours postnursing, but in the one strain tested (Sprague-Dawley) pituitary restoration to original level was not accomplished even by 9½ hours postnursing.

Discussion. The rapidity in which the act of nursing elicited a drop in pituitary lactogenic hormone in the present study would seem to substantiate the view such release is mediated through nervous reflex. Previous workers(2,3) allowed nursing to continue at least 3 hours before killing the mothers, though it has been our experience young of lactating rats become satiated within 30 minutes and stop sucking(4). Restoration of lactogen to prenursing levels was not accomplished even by 9½ hours postnursing, *i.e.*, the same time allowed for determination of prenursing levels. Lactating rats, of course, nurse more frequently than once every 20 hours on the 14th day of lactation(5) so pituitary lactogen values probably do not represent those found in normal nursing situations. The evidence obtained in both strains that pituitary lactogen had returned

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† Postdoctoral Fellow of NIH. This investigation supported in part by research grants from Public Health Service.

TABLE I. Comparison of Lactational Performance of 2 Strains of Albino Rats, Each with a Litter of 6 Young on 14th Day Postpartum.

	No. of rats	Avg wt of Litters (g)	Avg wt of Mothers (g)	Avg wt of Pituitary glands (mg)	Avg wt of Pituitary glands (mg/100 g)	Avg prenursing pituitary lactogen (Reece-Turner units/100 g)
Wistar-Missouri	40	140.3	214.9	8.8	4.1	3.50
Sprague-Dawley-Rolfsmeyer	60	145.8	290.2	10.2	3.5	2.44

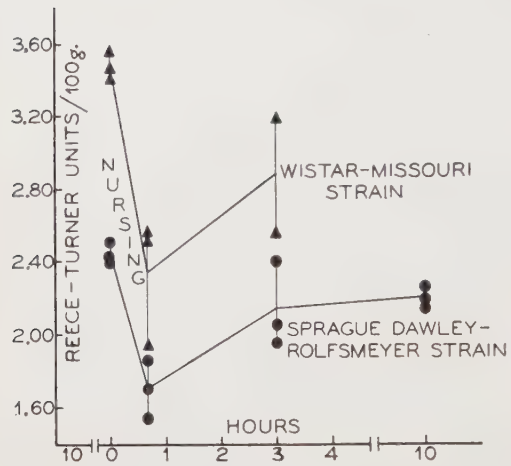

FIG. 1. Strain difference in release and restoration of pituitary lactogen in response to nursing stimuli after 10 hr isolation of mother and young in 14 day postpartum lactating rats. Each point represents assay of 5 pituitary glands.

in 2½ hours to about one-half that determined before nursing suggests enough hormone is restored to insure continuance of milk secretion following the next nursing. As lactation progresses it is possible there is a gradual diminution in amount of hormone restored after nursing to the point when milk secretion is no longer possible.

The observation that small Wistar rats are capable of raising litters as heavy as the larger Sprague-Dawley animals suggests more milk is produced in relation to body size in the Wistar rat. This may be due to the relative greater size of their pituitary glands and subsequent relative higher lactogen content. Also, the amount of hormone released in response to sucking was 33% greater in Wistar lactating rats. However, as pointed out by Folley and Young(6), maintenance of milk secretion is probably a response to the coordinated actions of a complex of pituitary hormones with lactogen as an essential element so it is difficult, therefore, to compare milk secretory activity on the basis of lactogenic hormone alone.

Summary. 1) The effects of nursing stimuli upon release and subsequent restoration of lactogenic hormone from the hypophysis has been studied in 2 strains of lactating rats on 14th day postpartum. 2) Wistar lactating rats produced litters which, when 14 days old, were almost as heavy as those of the much larger Sprague-Dawley mothers. There was 15% more pituitary gland/100 g in Wistar rats which, after 10 hours isolation of mother and litter assayed 30% more lactogen than pituitaries of Sprague-Dawley mothers (3.50 and 2.44 units/100 g, respectively). The Reece-Turner pigeon intradermal method of assay was used. Following 30 minutes nursing there was a greater discharge of lactogen (33%) in Wistar (1.16 units/100 g) than in Sprague-Dawley rats (0.78 unit/100 g). About one-half prenursing level was restored in both strains within 2½ hours postnursing but in one strain tested (Sprague-Dawley) restoration to prenursing level did not occur even 9½ hours postnursing. The influence of amounts of lactogen released in the two strains is discussed in relation to lactational performance.

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The Effects of Diamox® on the Uterine Response of Estrogen Treated Rats.* (23589)

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The earliest detectable response of the rat uterus to estrogen administration is a rapid imbibition of water, followed by a period of cellular proliferation and growth. The tissues of the uterus become edematous and the entire organ enlarges as a result of luminal fluid accumulation.

The drug, Diamox® (2-acetylamide-1,-3,4-thiadiazole-5-sulfonamide) is a powerful inhibitor of the enzyme carbonic anhydrase (1,2). Such inhibition in animals results in the excretion of alkaline urine, diuresis and lowered plasma bicarbonate(3). Perlmutt and Olewine(4) reported that acetazolamide increased the excretion of water about one and one-half times in normal rats. This greatly increased water excretion suggested that Diamox® may also affect the water imbibition of the uterus following administration of estrogen.

Materials and methods. The effects of Diamox® on uterine response to estrogen were studied in both sexually immature and adult rats. The effects on the immature rat uterus were determined by means of the bioassay method of Astwood(5). Animals 21-23 days old and weighing 40 to 50 g were given a single subcutaneous injection of 0.1 μg of estradiol-17β and graded doses of the sodium salt of Diamox®. The uteri were removed at various time intervals, stripped of fat and weighed immediately. Adult female rats 100 days old were used to determine changes in wet weight, dry weight and volumes of luminal fluid induced by Diamox®. Forty-eight animals were castrated and allowed to remain untreated for 7 days, at which time they were divided into 5 groups and were treated as follows: the first group received no treatment; the second was given 5 μg estrone daily for 3 days; the remaining 3 groups received 5 μg of estrone per day for 3 days and a daily injection of 40, 60 and 80 mg of Diamox®, respectively. The amount of luminal fluid was measured by withdrawing the fluid into a glass syringe fitted with a No. 20 needle. The exact amount of fluid could not be measured by this method, but a fair indication of the relative amount could be ascertained. *Water content* was determined by oven-drying uteri for 24 hours at 90-100°C. Nitrogen content of the dried uteri was measured by Nesslerization after digestion in a selenium-sulfuric acid mixture. The *sodium salt* of Diamox® (Lederle lot No. 7-6015)§ was dissolved in physiological saline at a concentration of 200 mg per ml. pH of this solution was 9.1. Estradiol-17β and estrone were dissolved in sesame oil. Rats were maintained on a normal diet and given water *ad libitum*. All injections were made subcutaneously either in the scruff of the neck or the skin of the back. The volume never exceeded 0.5 ml.

Results. The experiments in which the

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§ Diamox supplied through the courtesy of American Cyanamid Co., Research Division, Pearl River, N. Y.

TABLE I. Effect of Graded Doses of Diamox® on Uteri of Estradiol-Treated Immature Rats.

Treatment	% water*		Dry wt* (mg)		Nitrogen (mg/uterus)		No. animals
	6 hr	30 hr	6 hr	30 hr	6 hr	30 hr	
None	81.2 ± 1.3		4.0 ± .8		.52		25
0.1 μg estradiol	87.2 ± .8	84.2 ± 1.6	4.8 ± .6	5.4 ± 1.0	.56	.63	23
Idem + 20 mg Diamox	83.7 ± 1.6	83.3 ± .7	4.1 ± 1.0	6.2 ± 1.1	.47	.74	12
" + 30 " "	85.4 ± 1.3	83.9 ± 1.4	3.6 ± .7	5.3 ± .7	.50	.65	12
" + 40 " "	82.2 ± 1.9	83.4 ± 1.1	4.4 ± .6	5.2 ± .6	.53	.60	12
" + 50 " "	80.8 ± 2.4	82.7 ± 1.4	4.2 ± .5	5.2 ± 1.0	.49	.62	12

* Mean ± stand. dev.

immature animals were used (Table I) show that acetazoleamide is effective only in blocking uterine imbibition of water at the sixth hour, at which time it normally attains a maximum following injection of 0.1 μg estradiol. The growth phase, as measured by dry weight and nitrogen content at the thirtieth hour, is not disturbed by Diamox®. It is further interesting to note that in the 30 hour experiments the percentage of water is in all cases the same, the difference between any two groups not exceeding the standard deviation of the smaller. Values reported in Table I indicate that the percentage of water at the sixth hour does not vary inversely with dosage of Diamox®. The uteri of control animals injected with 20 mg of Diamox® per day for 3 days show no deviation from the normal at either the sixth or thirtieth hour.

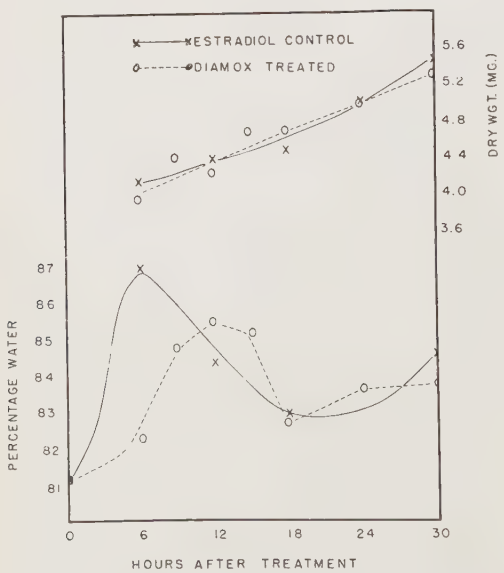

FIG. 1. Relationship of time to effects of Diamox® (40 mg) on uterine response to estradiol (.1 μg) stimulation.

Although Diamox® inhibited fluid imbibition which normally reached a maximum 6 hours after the injection of 0.1 μg of estradiol, further investigation showed that actually the response was only delayed and occurred at the twelfth hour (Fig. 1). A second injection of Diamox® on the sixth hour did not bring about a further postponement of the reaction. However, in all instances the response was somewhat less at 12 hours when Diamox® was given than at 6 hours when only estradiol was injected. In these experiments, as in the others reported (Table I), Diamox® did not modify uterine growth as shown by insignificant differences in dry weight at 30 hours.

In tests in which adult animals were given 3 daily injections of estrone or estrone plus Diamox® (Table II) the luminal fluid was withdrawn before the uteri were removed. Consequently, the percentage of water recorded was that of uterine tissue. Each group initially contained 8 animals but some were rejected due to mishaps in operational procedure; however, none died as a result of treatment.

Daily doses of 40 and 60 mg Diamox® given concurrently with 5 μg of estrone did not alter the percentage of water in the uterine tissues from that reported for the estrogen alone. However, 80 mg of Diamox® under the same conditions appeared to depress both uterine wet and dry weights. There also was a reduction in uterine luminal fluid as the dosage of Diamox® was increased. When Diamox® alone was administered to castrated females in doses of 60 mg per day for 3 days the percentage

TABLE II. Effect of Graded Doses of Diamox® on Uteri of Estrone-Treated Mature Castrated Rats.

Treatment*	Wet wt† (mg)	Dry wt† (mg)	% water†	Luminal fluid (ml)	No. animals
None	112.9 ± 4.0	20.9 ± 1.0	80.4 ± .7	Unmeasurable	8
5 μg estrone	218.9 ± 10.2	45.5 ± 3.2	80.5 ± .5	.6	7
Idem + 40 mg Diamox	189.8 ± 14.0	37.9 ± 2.2	81.1 ± .2	.4	5
" + 60 " "	218.3 ± 10.7	39.6 ± 1.5	80.5 ± .5	.2	6
" + 80 " "	172.5 ± 10.5	33.6 ± 1.6	80.9 ± .5	.2	7

* Animals inj. daily for 3 days. † Mean ± stand. dev.

uterine water was 79.6 ± 0.5, which was not significantly different from that indicated for untreated castrates (Table II).

Analyses of the ionic content of uterine fluid under these conditions showed no differences between estrone treated animals and those given both estrone and Diamox®. The sodium concentration in luminal fluid from animals given estrone was 121 mEq/l and potassium 42 mEq/l, while for those given estrone and Diamox® it was 122 mEq/l and 44 mEq/l, respectively.

Discussion. It is known that Diamox® causes an increase in excretion of water and an elevation of bicarbonate in the urine(3). Associated with this condition when Diamox® and estrogen were injected simultaneously, is a failure of the imbibition of fluid by the uterus during the first 6 hours. However, the effect of estrogen is only postponed as an accumulation of fluid, though somewhat reduced, occurs at about the twelfth hour. This shift in time of fluid imbibition does not influence the growth response of the uterus as seen at 30 hours.

The postponement of fluid imbibition from the sixth to the twelfth hour when Diamox® is given with estrogen is also a point of interest. If Diamox® is eliminated from the body more rapidly than estrogen an effective amount of estrogen may be present for a longer period than an inhibitory amount of Diamox®. However, a second dose of Diamox® administered on the sixth hour does not inhibit the delayed response of the twelfth hour nor does it modify uterine growth. This failure of a second dose of Diamox® to inhibit fluid imbibition is more difficult to explain but it may be that the animals become so acidotic they are unable to respond to a second injection.

There is also a question as to whether Diamox® produces its effect by a direct action upon the uterus. Mediation through carbonic anhydrase inhibition is doubtful; however, since Lutwak-Mann(6) has reported that the uterus of the rat does not contain a detectable amount of this enzyme. It is more probable that the effects of Diamox® as seen in the uterus are due to the general diuretic response of the animal.

Summary. Diamox® (40 mg) postpones the peak of water imbibition in the uterus of estradiol treated immature rats from the sixth to the twelfth hour. It is, however, incapable of blocking the normal growth response occurring at 30 hours. Daily doses of 40 and 60 mg of Diamox® given concurrently with 5 μg of estrone per day for 3 days to castrated adults did not alter the percentage of water in the uterine tissues from that reported for the estrogen alone. It is suggested that imbibition of water and the proliferative phase observed in the uterus under estrogen stimulation represent independent reactions.

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Induction of Lactation in Rabbits with Reserpine.* (23590)

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Sawyer(1) reported that when estradiol benzoate was injected subcutaneously into mature female rabbits for 2 days followed by a single intravenous injection of reserpine, milk was observed one week later in the lactiferous ducts of 15 out of 20 animals. Gaunt (private communication) has confirmed this finding, although in some of the rabbits only a pale watery secretion was seen. These observations suggested that reserpine may induce an increased secretion and/or release of prolactin from the anterior pituitary. In view of evidence that estrogen alone can increase pituitary prolactin content and initiate lactation in a number of species, including rabbits(2,3), it was of interest to determine whether the lactation induced in rabbits was in fact elicited by reserpine and not by the prior treatment with estrogen. It was also of interest to see whether reserpine could stimulate mammary development beyond that produced by estrogen alone.

Methods. Twenty-four mature female rabbits, predominantly New Zealand White in origin, were used in 3 separate experiments. These rabbits weighed 2.8-4.8 kg each and were fed a standard rabbit diet. In the first experiment, 8 rabbits were injected subcutaneously for 2 successive days with 0.2 mg estradiol‡ daily, and on the third day 4 were injected intravenously with 1 mg reserpine/kilo body weight. The other 4 served as controls. One week later a slight amount of milk could be expressed from the nipples of 3 of the 4 reserpine-injected rabbits and from none of the controls. Milk could still be expressed from one of the reserpine-treated rabbits almost one month after administration of the drug. In the *second experiment,* 4 rabbits were made pseudopregnant by a single intravenous injection of chorionic gonadotrophin, and 15 days later 2 of these rabbits were injected once intravenously with 1 mg reserpine/kilo body weight. The other 2 served as controls. Only the 2 reserpine-injected rabbits had milk when examined 5-7 days later, and the quantity of milk present in the mammary glands appeared to be somewhat greater than in the rabbits treated with the drug in the first experiment. Lactation persisted in one of the 2 reserpine-injected rabbits for more than a month after treatment. In the *third experiment,* 12 rabbits were injected with 0.2 mg estradiol daily for 10 days, and on the 11th day 6 rabbits were injected intravenously with the same dose of reserpine used in the previous 2 experiments, and the other 6 served as controls. One week later all the rabbits were killed and their mammary glands were removed for macroscopic and histological examination. Their pituitaries were also removed for prolactin assay. *Lactation* occurred in all 6 reserpine-treated rabbits but not in the controls. When the mammary glands were exposed it was apparent that those from the reserpine-treated rabbits were enlarged and milk could be seen in the lactiferous ducts (Fig. 1). On the other hand the mammary glands of the controls were so undeveloped that they were not readily visible macroscopically. Stained whole mounts of these mammary glands showed that when estrogen was followed by reserpine, there was extensive development of the lobule alveolar system comparable to that seen in pseudopregnant rabbits. Estrogen alone elicited mainly an extension of the duct system (Fig. 2). Histological examination revealed that the mammary alveoli of reserpine-treated rabbits were filled with secretion, whereas few alveoli were seen in controls and the ducts were dilated. A fuller description

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† We are indebted to Dr. Robert Gaunt of Ciba Pharmaceutical Products, Summit, N. J., and to Dr. Russel Kraay of Eli Lilly and Co., Indianapolis, Ind., for the reserpine used.

‡ The estradiol was kindly furnished by Dr. J. O. Reed of Foundation Labs., New York City.

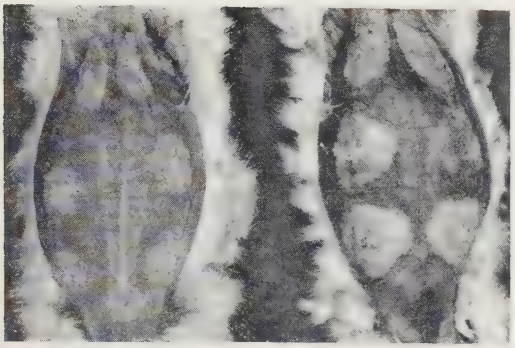

FIG. 1. Lactating mammary glands from 2 rabbits injected subcutaneously for 10 days with 0.2 mg of estradiol daily followed by a single intravenous injection of 1 mg reserpine/kilo body wt. The mammary glands of rabbits injected only with estradiol were not visible macroscopically.

of the histology of the mammary glands as well as the results of the prolactin assays will be published upon completion of this phase of the work.

Discussion. The results reported here confirm the findings of Sawyer(1) and Gaunt (private communication). Experiments currently in progress also indicate that daily administration of reserpine can initiate milk secretion in mature female rabbits without prior treatment with estrogen or induction of pseudopregnancy. There can be little question that reserpine can elicit mammary growth in addition to initiating lactation in rabbits. In none of the reserpine-treated rabbits was the degree of lactation induced comparable to that seen in rabbits after parturition. It is possible, however, that daily administration of reserpine to rabbits with fully-developed mammary glands would produce a much greater yield of milk than seen in the present study.

The mechanism(s) whereby reserpine initiates lactation in rabbits remains to be elucidated, although the results suggest that release and/or secretion of prolactin by the anterior pituitary are increased by the drug. Other factors from the pituitary favorable to mammary growth and lactation may also be released at increased rates by reserpine, although prolactin has been shown to be the only hormone capable of directly stimulating the mammary secretory cells to produce milk(4). That reserpine may induce an increased release of prolactin from the pituitary is also suggested by the observation of Barraclough(5) that reserpine injections into rats during diestrus result in a maximum and prolonged decidual response following traumatization of the uterus.

It is of interest that several clinical investigators have recently observed that administration of chlorpromazine occasionally elicits lactation in women patients(6-9). A similar mechanism(s) to that of reserpine is probably involved. That reserpine may inhibit rather than stimulate other functions controlled by the endocrine system is suggested by reports that it may depress thyroid function(10) and interfere with the estrous cycle and conception in the rat(11,12). There is also evidence that reserpine may inhibit certain hypothalamic functions(13,14).

Summary. 1. Twenty mature female rabbits were injected subcutaneously with 0.2 mg estradiol daily for 2 or 10 days, followed by a single intravenous injection of 1 mg reserpine/kilo body weight into 10 animals. The other 10 rabbits served as controls. Nine of the 10 reserpine-treated rabbits came into lactation one week later while none of the controls produced milk. Four other rabbits were made pseudopregnant, and 15 days later 2 were injected as above with reserpine and 2 served as controls. Only the 2 reserpine-treated animals came into milk. 2. Macroscopic and histological examination of the mammary glands of reserpine-treated rabbits

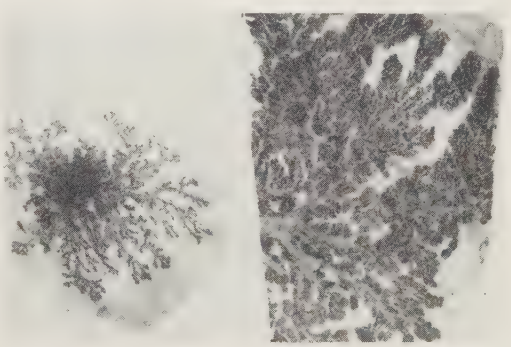

FIG. 2. *Left.* Whole mammary gland from a rabbit injected with estradiol for 10 days and killed 1 week later. *Right.* Half of mammary gland from a rabbit injected with estradiol for 10 days, followed by a single injection of reserpine and killed 1 week later.

previously injected with estrogen revealed extensive lobule-alveolar development with secretion in the alveoli, whereas controls injected only with estrogen showed an extension of the duct system with no alveolar growth. 3. It is probable that reserpine induced an increased secretion and/or release of prolactin and perhaps of other hormones favorable to mammary growth and lactation, but confirmation of this hypothesis awaits assay of pituitary hormones.

Addendum: Pituitary assays now completed reveal that reserpine induced a substantial increase in prolactin in both normal and estrogen-treated rabbits.

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Effects of Growth Hormone on Lactation and Body Growth of Parturient Rats.* (23591)

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Growth hormone (GH) has been demonstrated to increase milk yields in the parturient cow(1,2), sheep(3) and goat(4). Shaw *et al.*(5) reported that when GH was injected into cows on a limited ration, milk production fell for the first 3 days but this was followed by a return to normal levels of milk together with a striking increase in milk fat. This suggests that GH injections into lactating ruminants may preferentially divert nutrients to the mammary glands rather than be used for growth or maintenance purposes. Significant effects of GH on body weight of ruminants have not been reported.

It was of interest to determine whether injections of GH into parturient rats would (a) elicit increases in milk production similar to those observed in ruminants and (b) increase body weight. The ability of GH to increase body weight in mature female rats which have reached growth stasis constitutes an established assay method for this hormone(6).

Methods. Mature female albino rats (Carworth) of uniform age and weight and which had reached growth stasis were bred, and on the day of parturition their litters were reduced to 6 young per dam. On the 5th day after parturition, 20 rats were divided into 2 groups of 10 each. Ten were injected subcutaneously with 1 mg GH† daily in .1 cc distilled water, and the other 10 rats (controls) were injected only with the same volume of distilled water. This treatment was continued until the 17th day after parturition. The rats were maintained in an air-conditioned, temperature controlled room, and both dams and litters were weighed

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† A gift of growth hormone was kindly supplied by The Endocrinology Study Section, N.I.H.

TABLE I. Effects of GH on Average Body Weight of Suckled Rats and Litters.

Treatment	Avg body wt (g) on following postpartum days— 1	3	5	7	9	11	13	15	17	Avg wt gain (g), 5th-17th day
Controls										
Dam	268 ± 5.4†	267	264	264	266	268	267	265	264	0 ± 3.5†
Litter of 6	38 ± 1.3	51	69	87	107	129	150	170	186	117.4 ± 5.8
GH*										
Dam	265 ± 5.4	262	265	271	273	280	283	285	288	23.0 ± 3.1
Litter of 6	37 ± .3	49	65	82	105	125	147	170	191	125.5 ± 4.0

* GH started on 5th postpartum day. † Stand. error of mean.

every 2 days.

Results. The mother rats and litters of both groups initially weighed about the same for the first 5 days after parturition (Table I). Injections of GH beginning on the 5th postpartum day induced a steady increase in body weight over the controls. At the end of the 17th day the GH-treated rats had gained an average of 23.0 ± 3.13 g each, or about 2 g per day, while the controls showed no significant change in body weight. Each of the GH-injected rats gained from 10 to 36 g during the experimental period, whereas 5 controls either lost or failed to gain in body weight. The litters of both groups grew at about the same rate during the experimental period, indicating that GH did not enhance milk production.

Discussion. These results suggest that a dose of GH sufficient to induce a significant increase in body weight of suckled mother rats which had reached growth stasis, does not increase milk yield as judged by growth of their litters. Apparently GH is not galactopoietic in the rat in contrast to its action in the cow, sheep and goat. Work in our laboratory has recently demonstrated that injections of prolactin, ACTH or cortisone into parturient rats can increase growth rate of litters(7,8). Furthermore, these hormones were able to partially prevent involution of the mammary parenchyma of parturient rats after their young were removed, whereas GH had no apparent effect on mammary involution.

Several workers have demonstrated that lactation can be initiated in hypophysectomized guinea pigs with a combination of prolactin and ACTH or adrenal cortical hormones(9,10). Lyons (private communication) recently stated that only prolactin and ACTH were essential to initiate lactation in hypophysectomized rats with developed mammary glands. Thus, despite the demonstrated galactopoietic activity of GH in the cow, sheep and goat, it does not appear to be galactopoietic or an essential factor for either the initiation or maintenance of lactation in the rat.

Summary. Twenty mature female albino rats which had reached growth stasis were bred, and on the day of parturition their litters were reduced to 6 young each. On the 5th postpartum day 10 rats were injected with 1 mg GH daily for 12 days and the other 10 rats served as controls. The GH-injected rats gained an average of 2 g each daily during the experimental period whereas the controls showed no increase in body weight. The growth rate of the litters was the same in both groups, indicating that GH did not increase milk production. This contrasts with the demonstrated ability of GH to increase milk production in cattle, sheep and goats.

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Biochemical Changes in HeLa Cells Associated with Infection by Type 2 Adenovirus. (23592)

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Infection of tissue culture cells with poliomyelitis virus has been shown to result in a rapid alteration of cellular metabolism, with temporary increased production of lactic acid and suppression of uptake of radioactive glycine(1). In their overall effects on HeLa cells, adenoviruses present a marked contrast to the poliovirus system previously studied; whereas poliovirus appears to suppress cellular metabolism with overall inhibition of acid production(2), adenoviruses stimulate acid production by infected HeLa cells(3,4) and do not interfere with the incorporation of vital stains for long periods after complete cytopathic effects(5). Consequently, experiments were conducted to determine some of the biochemical changes accompanying adenovirus infection of HeLa cells. A high multiplicity of virus to cells was employed in order to obtain as nearly synchronous infection as possible. Experiments were designed to survey a broad area of biochemical activity, with more detailed observations to follow upon any positive observations. This report will deal with the effect of the time course of the infection on the production of lactic acid by HeLa cells, and on the rate of their uptake of P^{32} and glycine-2-C^{14}, as well as with the distribution of these isotopes among several biochemical fractions after 5 hours of infection.

Materials and methods. HeLa cell cultures. HeLa cells† were grown in 2 oz. or 32 oz. prescription bottles, in a medium consisting of 10% human serum in Eagle's basal medium (BME)(6). In the majority of experiments, all cultures were prepared from a single pool of cells, the tissues were re-fed at 2 or 3 days, and the cultures used after an additional 2 days; only cultures with confluent cell sheets were used. Immediately before virus inoculation, the growth medium was removed and the cultures were rinsed with BME or 3:1 Hanks-Simms solution(7, 8). The 2 oz. bottle cultures were washed 3 times with 5 ml, and the 32 oz. cultures 2 or 3 times with 20 ml. Maintenance medium was 5% chicken serum in Eagle's basal medium, in a volume of 5 ml for the 2 oz. bottles and 40 or 50 ml for the 32 oz. bottles. All media contained penicillin, 250 u/ml, and streptomycin, 250 μg/ml. *Virus.* The Ad. 6 strain of adenovirus type 2, was used throughout. Virus pools were prepared in HeLa or KB cell(9) cultures: 4 pools were used, each of which had an infectivity titer for HeLa cells of $10^{8.5}$ $TCID_{50}$ per ml. The 2 oz. bottle cultures received 0.2 ml, and the large bottle cultures 1.6 ml of the undiluted virus suspension. This dose of virus produced complete cytopathogenic effects with beginning loss of cells into the culture medium within 18 to 24 hours after inoculation of the HeLa cell cultures. Control cultures were inoculated with an equal volume of medium from uninfected cultures of the same type as were used for production of the virus. Both virus and control inocula were clarified by centrifugation at 2,000 rpm for 10 minutes. *Radioactivity.* Radioactive inorganic phosphate was received from the Atomic Energy Commission, Oak Ridge, Tenn. Glycine-2-

* Lab. of Infect. Dis.

† Seed cultures were obtained from Microbiological Associates, Bethesda, Md.

C^{14} was purchased from Tracerlab, Inc., Boston, Mass. The amino acid generally had a specific activity of 200 μc in about 14 mg of compound. Aliquots of the material to be counted were deposited into weighed stainless steel planchets, dried, reweighed and counted. Where necessary, corrections were made for self absorption of the C^{14}. In some experiments both P^{32} and glycine-2-C^{14} were added to the same tissue culture. In such cases, the combined P^{32} counts and C^{14} counts of the sample were determined first and then the counts capable of penetrating an absorber of 24.2 mg/cm^2 were measured. This latter absorber permitted about 80% of the beta particles from P^{32} to come through with virtually none of the beta particles from C^{14} penetrating. By suitable calculation, the counts per minute for P^{32} and C^{14} were obtained. *Lactic acid* was determined by the method of Barker and Summerson(10). *Nucleic acids* were determined by the Schneider method(11) using diphenylamine as the chromogenic agent for DNA(12), and phloroglucinol(13) for RNA. *Phosphorus:* The method of Gomori(14) was used. *Two basic types of experiment* were performed. In the first type, the effect of infection of HeLa cells with type 2 adenovirus on the uptake of glycine-2-C^{14}‡, on the uptake of P^{32} and on the production of lactic acid was studied at selected times after infection. In the second type the incorporation of the isotopes into several biochemical fractions of infected and control cells was compared after 5 hours of infection. Four experiments of the first type were performed, and 3 of the second. The results of all experiments were essentially in agreement, with the three fractionation experiments furnishing additional confirmatory evidence for the experiments dealing with isotope uptake by whole cells. One experiment of each type will be described in detail. In order to indicate the extent of variation from one experiment to the next, data of another experiment, similar except for minor details, will also be presented.

‡ "The uptake of glycine-2-C^{14}" in the text, means uptake of the radioactivity of glycine-2-C^{14} and not necessarily the uptake of the intact compound.

Results. Effect of type 2 adenovirus on metabolic activities of HeLa cells during 96 hours of infection. The medium was poured off from one hundred twenty 2 oz. prescription bottles containing a fairly heavy continuous sheet of HeLa cells. The cells were washed 3 times with 2 ml of medium and then fed with 5 ml of maintenance medium. To half the flasks was added 0.2 ml of virus, representing about $10^{7.8}$ $TCID_{50}$. The ratio of virus to cells was about 70. To the other flasks, which served as controls, was added 0.2 ml of medium of the type in which the virus was suspended. This medium had served as nutrient for HeLa cells for a few days in order to have it comparable to the virus inoculum. The bottles were placed at 37°C and allowed to incubate. Groups of 8 infected bottles and 8 control bottles were preselected for removal after increasing periods of time. Thirty minutes before a given group of infected and control bottles were scheduled to be removed, they received 0.2 ml of a solution containing 0.1 μc of glycine-2-C^{14} and 0.1 μc of inorganic P^{32} in saline. When these bottles were taken for analysis, the growth medium was poured off, the cells were scraped into the medium with a rubber tipped policeman, like suspensions were pooled, the cells centrifuged out and washed 4 times with 40 ml each time of 0.85% NaCl in the cold. The medium was frozen and later used for lactic acid analysis. The packed cells were suspended in 0.5 ml of saline and transferred to stainless steel planchets, dried for 2 hours at 130°C, and weighed. The observed weight was corrected for the weight of NaCl arising from the saline to give the net weight of cells. The amount of radioactivity, as C^{14} and as P^{32} was determined as described under methods, and the data expressed as cpm/mg dry weight of tissue. The data for the uptake of the radioactivity of glycine are summarized in Fig. 1 and for the uptake of P^{32} in Fig. 2.

Since in all cases the radioactive solutions were in contact with the cells only for the 30 minute period just prior to their removal, what is shown in Fig. 1 and 2 is the effect of time of infection on the rate of uptake of the isotope, not on the cumulative uptake.

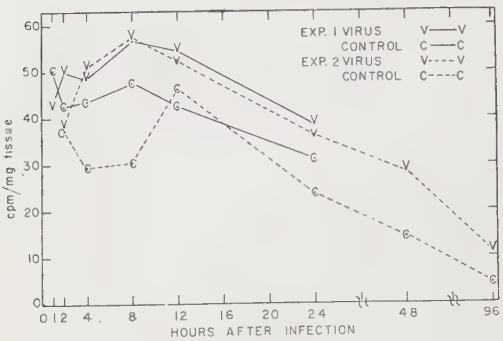

FIG. 1. Effect of type 2 adenovirus on uptake of radioactive glycine by HeLa cells.

It will be seen that early in the infection, and before the time when new adenovirus is thought to be produced(5,15), there was a definite increase in the uptake of P^{32} and C^{14}. This stimulative effect of the virus was still manifest after 48 hours and possibly longer.

The effect of virus infection on the glycolysis of the cells was determined in the following way. The amount of lactic acid present in the fluid immediately after addition of virus (zero time) was found by taking 8 infected and 8 control bottles at the time of infection and removing cells as described above. Lactic acid content was determined in these fluids. This was used as a blank figure to subtract from the amount of lactic acid found in the samples taken off after varying periods of infection. The net amount of lactic acid so calculated represents the amount of lactic acid accumulated in the fluids in the period of time subsequent to the initiation of the experiment. The data from the experiment described here as well as those of another experiment are represented in Fig. 3, where the accumulated lactic acid per mg of cells is plotted against time after addition of the inoculum.

An increased lactic acid accumulation was always found in the infected cells as compared with controls. In the experiments shown in Fig. 3, this rise was apparent after 20-24 hours, but in other experiments it was seen as early as 8 hours after infection. It will be noted that in Exp. 3 there was a marked rise in lactic acid accumulation after 24 hours of incubation, while such rise was not seen in Exp. 1. Since the glucose of the original medium is probably used up by 24-36 hours, one possible interpretation of the difference between the two experiments is that the cells used in Exp. 3 had the capacity to produce lactic acid from some other substrate, while those of Exp. 1 either did not

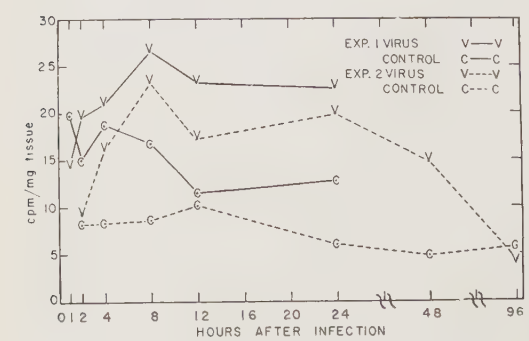

FIG. 2. Effect of type 2 adenovirus on uptake of radioactive inorganic phosphate by HeLa cells.

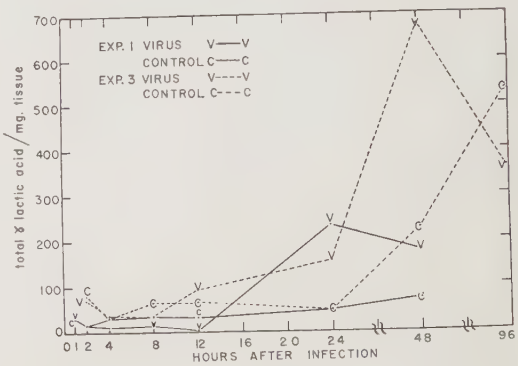

FIG. 3. Effect of type 2 adenovirus on lactic acid production by HeLa cells.

or destroyed the lactic acid so formed more rapidly. However, we have no evidence on this point.

Biochemical fractionation. The data above deal with the uptake of radioisotope by the whole cells; this uptake is of course a reflection of a weighted average of the uptakes by the different biochemical fractions. We studied the effect of infection on the uptake of the radioisotopes by several gross biochemical fractions.

The medium was poured off twenty 32 oz. Blake bottles containing solid sheets of HeLa cells. The cell sheet in each bottle was washed twice with 20 ml of Hanks-Simms solution, and 40 ml of Eagle's basal medium

containing 5% chick serum were then added. To half of the bottles were added 1.6 ml of virus and to the other half 1.6 ml of used growth medium similar in composition to that of virus inoculum. The virus/cell ratio was about 50. The bottles were placed at 37°C for 4½ hours, at which time half of the infected and half the control bottles received 2 μc of glycine-2-C^{14} and the other half received 8 μc of P^{32}. The 20 Blake bottles were thus divided as follows: 5—infected cells + P^{32}; 5—control cells + P^{32}; 5—infected cells + glycine-2-C^{14}; 5—control cells + glycine-2-C^{14}. At the end of an additional 30 minutes the fluid was poured off and the cells were scraped into 20 ml of 0.85% NaCl. Comparable cells were combined and centrifuged lightly in the cold. The cells were washed by centrifugation 3 additional times with 40 ml each time of cold 0.85% NaCl. The packed cells were made up in 0.85% NaCl to 10 ml, 1 ml was plated into weighed stainless steel planchets, while another 1 ml was taken off and stored in a freezer for the determination of total phosphorus. To the remaining 8 ml of cells was added 0.8 ml of 100% trichloracetic acid solution, and the flasks were allowed to stay overnight in the refrigerator. This and subsequent treatment of the cells is described qualitatively in the accompanying flow sheet, along with the analyses performed on the indicated fraction.

The data from 2 experiments are summarized in Tables I, II and III. In Table I are given the data relevant to phosphorus quantitation and to P^{32} uptake; in Table II those relevant to uptake of the radioactivity of glycine-2-C^{14}; in Table III, the individual and averaged values of RNA and DNA concentration.

In all 3 experiments, uptake of P^{32} into all fractions (with the occasional exception of lipid) was stimulated by the virus. On the other hand, the stimulatory effect of virus on the uptake of glycine-2-C^{14} by the cells was actually a reflection of the increased radioactivity in the acid soluble fraction, and the other 3 fractions were affected not at all or inhibited slightly. No reproducible effect of the virus on the concentration of P in any of the fractions was found, nor was any effect found on the concentration of RNA and DNA as determined by colorimetric methods for their specific sugars.

Discussion. The appearance of metabolic changes within 2 to 4 hours after inoculation is somewhat surprising in view of the reportedly slow rate of absorption of adenovirus and the 19 to 21 hour latent period before increase in intracellular virus(5,15).

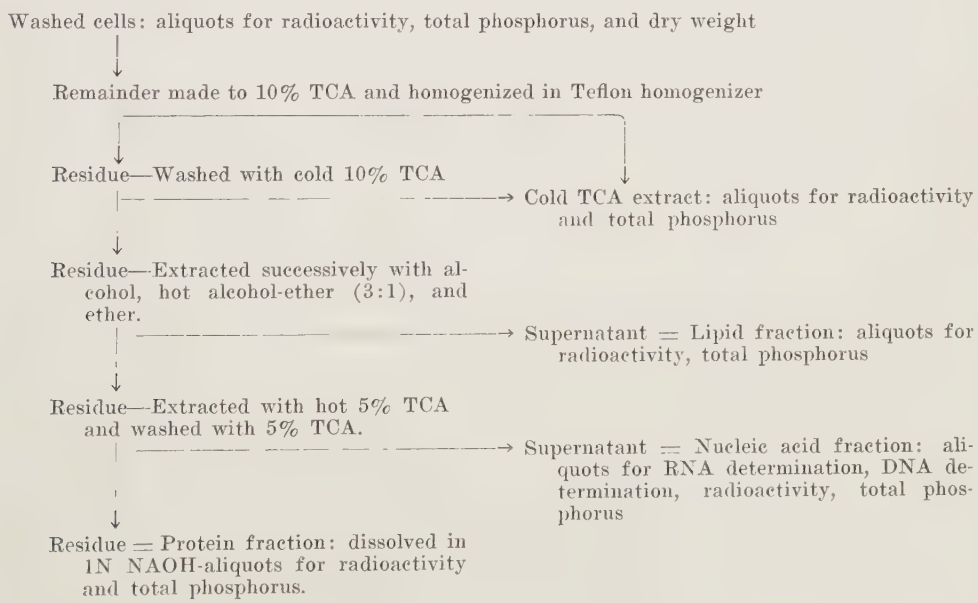

TABLE I. Effect of Type 2 Adenovirus on Phosphorus Uptake by Fractions from HeLa Cells after 5 Hours of Infection.

Fraction	Exp.	cpm/mg IV	cpm/mg V	Ratio Inf./Cont. IV	Ratio Inf./Cont. V	γP/mg IV	γP/mg V	cpm/mg P IV	cpm/mg P V
Whole cells	Infected	545	1114	1.18	1.57	15.9	17.0	34,270	65,500
	Control	461	708			14.4	17.6	32,010	40,200
Cold TCA	Infected	320	710	1.17	1.57	3.6	3.1	88,880	22,900
	Control	273	451			3.1	3.2	88,060	14,090
Lipid	Infected	5.2	12.7	1.00	1.30	2.7	3.6	1,962	3,520
	Control	5.2	9.7			2.9	3.5	1,793	2,770
Nucleic acid	Infected	86	74	1.26	1.68	5.2	6.9	16,538	10,700
	Control	68	44			5.9	5.3	11,525	8,300
Residue	Infected	25.1	38	1.78	1.90	6.6	.9	3,803	42,200
	Control	14.1	20			5.0	.7	2,802	28,500

However, since large doses of adenovirus comparable to those used in the present study can produce specific cytopathic effects within 2 to 4 hours after inoculation(16), it is apparent that adenoviruses can produce profound effects long before the appearance of infectious virus. That the observed metabolic changes were not simply the result of permeability changes is indicated by the markedly different patterns observed with phosphorus and glycine.

TABLE II. Effect of Type 2 Adenovirus on Uptake of Glycine-2-C^{14} by Fractions from HeLa Cells after 5 Hours of Infection.

Fraction		Exp. IV cpm/mg	Exp. IV Ratio Inf./Cont.	Exp. V cpm/mg	Exp. V Ratio Inf./Cont.
Whole cells	Inf.*	130.5	1.40	227	1.60
	C	93.0		142	
Cold TCA	Inf.	18.0	1.71	40	1.67
	C	10.5		24	
Lipid	Inf.	8.6	1.06	10	.9
	C	8.1		11	
Nucleic acid	Inf.	4.7	.80	3.6	.81
	C	5.9		4.4	
Residue	Inf.	54.0	1.05	82	.84
	C	51.2		97	

* Inf. = Infected; C = Control.

TABLE III. Effect of Type 2 Adenovirus Infection on Nucleic Acid Concentration of HeLa Cells; 3 Experiments. Figures expressed as % of dry wt.

	RNA, %	Avg, %	DNA, %	Avg, %
Infected	6.1, 4.7, 5.2	5.3	3.9, 3.7, 4.5	4.0
Control	5.5, 4.7, 5.9	5.3	3.5, 3.5, 4.8	3.9

A difficulty in relating the observed results to the growth cycle of the virus is the problem of obtaining synchronous infection of most of the cells in the culture. Although a high multiplicity of virus to cells was used, results of studies by other workers indicate that synchronous infection probably was not obtained; thus, Harford *et al.*(17), using the electron microscope, found virus particles in only 5 to 20% of HeLa cells infected with adenovirus. Droz and Chany,§ using autoradiographic technics, observed increased isotope uptake in only a small proportion of HeLa cells infected with a large dose of adenovirus. If only a small proportion of cells are affected metabolically, the degree of metabolic change is all the more striking, since the data obtained here show the net effect on the culture as a whole. It should be emphasized that the biochemical fractionations were performed only at 5 hours; it is quite possible that a different pattern would have been observed at a later time when infectious virus is being liberated.

The observed metabolic effects possibly could be associated with the early cytopathic changes seen when large doses of adenovirus are used, and both might, in turn, be attribu-

§ Personal communication.

table to the presence in the fluids of soluble antigens or other byproducts of virus multiplication. However, 3 preliminary pieces of evidence suggest that the early metabolic effects are the result of infection. First, stimulation of uptake of P^{32} was prevented by antiserum to type 2 adenovirus and not by normal rabbit serum. Second, a preparation of type 2 adenovirus whose infectivity had been destroyed by a combination of ultraviolet light irradiation and heating, but which still produced the early cytopathic changes, did not cause metabolic stimulation, whereas the untreated preparation produced the usual increase in turnover in the same test. Third, experiments with type 2 adenovirus in monkey kidney tissue culture, in which early cytopathic changes are not produced, gave metabolic results similar to those obtained in HeLa cells.

Some points of similarity and some points of difference might be pointed out in comparing the effects reported here with those found with type 3 poliovirus in monkey kidney tissue culture(1). In the latter system, an increased lactic acid production was manifest in the infected cultures within 1-2 hours after addition of the virus, which phenomenon was not seen with the adenovirus-HeLa cells until 8-24 hours. One might be inclined to associate this difference with the slower rate of absorption of adenovirus(5,18), but the effects of adenovirus on uptake of radiophosphate and radioglycine were seen within 2-3 hours.

Insofar as the uptake of radioglycine in the 2 virus-cell systems is concerned, some marked differences were noted. In the polio-kidney system, the uptake of radioglycine was strongly inhibited in all the fractions, and, consequently, in the whole cells. With the adenovirus-HeLa system, the uptake of radioglycine by the whole cells was stimulated, but this effect was restricted to the acid soluble subfraction, with the other fractions showing no effect or a slight inhibition. Experiments in these laboratories to determine the effects of poliovirus on the uptake of P^{32} by monkey kidney tissue culture cells have not given reproducible results, so no comparison can be made with such effects in the adenovirus-HeLa system. Preliminary experiments with poliovirus in HeLa cells suggest that its behavior there is similar to its behavior in kidney cells.¶

This group of experiments was designed to survey a broad area of metabolic activities to see if any effects of adenovirus could be found. Currently, investigations are under way to examine in more detail those effects that have been noted.

Summary. Infection of HeLa cells with a large inoculum of adenovirus type 2 produced a rapid and prolonged stimulation of cellular uptake of the radioactivity of inorganic P^{32} and glycine-2-C^{14}, with no change in concentration of P, RNA, or DNA. In fractions taken 5 hours after inoculation, the increased rate of P^{32} uptake was observed in each of the biochemical fractions tested, minimally in lipid. Glycine uptake was increased only in the acid-soluble fraction. In contrast to the early alteration of radioisotope uptake, increased production of lactic acid was not observed until 8 to 24 hours after infection.

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Incorporation of C^{14} of Acetate-1-C^{14} and Pyruvate-2-C^{14} into Brain Cholesterol in the Intact Rat. (23593)

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Srere *et al.*(1) found that slices prepared from newborn rat brain were able to convert acetate to cholesterol. With slices from adult animals, however, no formation of cholesterol from acetate could be detected. In the present work, the incorporation of labeled acetate and pyruvate into brain cholesterol was studied in the intact rat by injecting the labeled compounds into the cisterna magna. Under these conditions, appreciable incorporation of both acetate and pyruvate was observed in rats up to 1 year of age.

Methods. Male Sprague-Dawley rats were injected with sodium acetate-1-C^{14} (Tracerlab) and sodium acetate-1-C^{14} (Nuclear Instrument and Chemical Corp.) in isotonic saline by a technic previously described(2). Both compounds had a specific activity of 1 mc per millimole. Unless otherwise indicated, all injections were made under Nembutal anesthesia. A stock laboratory diet was provided *ad libitum* throughout the experiments. At stated intervals after injection, the animals were killed by decapitation. The brains were removed and saponified in alcoholic KOH for 4 hours. The saponifiable and unsaponifiable fractions were separated by extracting the mixture with petroleum ether, evaporating the extract to dryness, and then extracting the residue with hot ethanol, the procedure being similar to that described by Van Bruggen *et al.*(3). Each ethanol extract was made to a volume of 25 ml, and aliquots were used for the determination and isolation of cholesterol. The cholesterol was precipitated from the ethanol extract as the dibromide, regenerated by debromination with zinc dust, and recrystallized from cold methanol, as described by Schwenk and Werthessen(4). Suspensions of the purified product in a 4:1 methanol-water mixture were then deposited for counting on 5 sq. cm aluminum disks by centrifuging the suspensions in a special plating assembly(5). Ordinarily a deposit of about 5 to 10 mg was obtained. Some loss occurred in the plating process, owing to the small but appreciable solubility of cholesterol in the methanol-water mixture and to the tendency of some of the solid to cling to the surface of the supernatant fluid. The actual amount of cholesterol plated was determined in each case by weighing. All determinations of radioactivity were carried out in a windowless gas flow counter. Corrections for the self-absorption of cholesterol were made according to the data for wax(6). The cholesterol content of the ethanol extracts was found by the method of Sperry and Webb(7). Most of the results are expressed as the % incorporation of C^{14} into cholesterol, calculated as the total activity of the cholesterol found in the ethanol extract divided by 1% of the administered activity. Since some degradation of brain cholesterol is known to occur during saponification(8), the % incorporation figures thus obtained must be somewhat lower than the actual values.

Results. Comparison of intracisternal and intraperitoneal injections of acetate-1-C^{14}. After preliminary experiments had indicated that adult brain cholesterol could be labeled *in vivo* with acetate-1-C^{14}, an experiment was performed in which different routes of

TABLE I. Incorporation of C^{14} of Acetate-1-C^{14} into Brain Cholesterol following Intracisternal and Intraperitoneal Injections into Rats.*

Injection	Acetate inj./100 g body wt (μc)	Specific activity of cholesterol§ (c.p.m./mg)	% incorporation§
Intracisternal†	6.7	190	.05
Intraper.†	6.7	28	.002
" ‡	40	40	.006

* 200 to 250 g rats.
† Single inj.
‡ Given in 3 equal inj. at 3 hr intervals without anesthesia.
§ Each result represents avg of 2 animals.

administration were compared. As was to be expected, intracisternal injection proved much more effective than intraperitoneal injection in labeling brain cholesterol (Table I). It was found, however, that the rates of incorporation into liver cholesterol were substantially the same for the two routes of administration.

Effect of age on incorporation. The incorporation of the C^{14} of both the acetate and the pyruvate into brain cholesterol was much greater in young rats than in older animals (Fig. 1 and 2). It will also be noted that the C^{14} of the acetate was incorporated to a greater extent than that of the pyruvate. With 200 to 250 g rats (no curve shown), the % incorporation values of the C^{14} of labeled acetate fell about midway between the values for the weanling and the 1-year-old animals.

Effect of acetate on incorporation of C^{14} of pyruvate-2-C^{14}. The rats used in this experiment were divided into 4 groups and injected intracisternally with a fixed dose of labeled pyruvate together with graded amounts of unlabeled acetate. The results (Table II) indicate that acetate inhibits incorporation of the C^{14} of pyruvate-2-C^{14} into brain cholesterol.

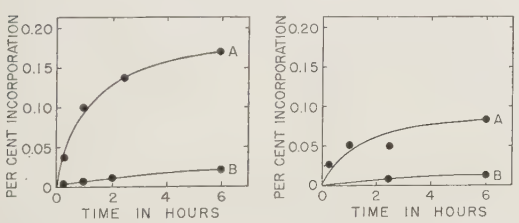

FIG. 1. (left). Effect of age on incorporation of the C^{14} of acetate-1-C^{14} into brain cholesterol. Each rat was injected intracisternally at zero time with 1.7 μc per 100 g of body weight. Each point represents the pooled brains of 2 animals. Curve A, weanling rats; curve B, 1-year-old rats (450-550 g).

FIG. 2 (right). Effect of age on incorporation of the C^{14} of pyruvate-2-C^{14}. Conditions are the same as those for acetate in Fig. 1.

TABLE II. Effect of Acetate on Incorporation of C^{14} of Pyruvate-2-C^{14} into Brain Cholesterol.

Rats weighing between 200 and 225 g were inj. intracisternally with 3.8 μc of labeled pyruvate plus the indicated amt of unlabeled acetate in .015 ml of solution. Animals were sacrificed 6 hr after inj.

No. of animals	Acetate inj. (μmoles)	Specific activity of cholesterol* (c.p.m./mg)
3	.0	73
1	.56	53
2	11.3	40
2	56.3	21

* Brains from animals in each group were pooled.

Time-course of labeled cholesterol. In this experiment, the specific activity of the brain cholesterol was followed for 72 days. After the initial rise, the activity remained fairly constant throughout the experimental period, as can be seen in Table III. No decline in activity was observed.

Discussion. The results indicate that the C^{14} of acetate-1-C^{14} and pyruvate-2-C^{14} is incorporated into the brain cholesterol of adult rats. That acetate is utilized directly by the brain for cholesterol formation is suggested by the fact that intracisternal injection produces much greater incorporation than intraperitoneal injection. Intracisternally administered acetate was also incorporated rapidly into liver cholesterol, but it seems unlikely that such cholesterol could have contributed

TABLE III. Time-Course of Labeled Brain Cholesterol.

Rats weighing between 200 and 250 g were inj. intracisternally with 6.7 μc of labeled acetate/100 g of body wt.

Time after inj. (days)	Specific activity of cholesterol* (c.p.m./mg)
2	420
14	540
28	520
49	515
70	560
72	550

* Each value represents avg of 3 rats.

appreciably to the labeled product found in the brain, since plasma cholesterol does not appear to be capable of penetrating the brain cells(9). The failure of labeled cholesterol to show a decline in activity during the time-course experiment (72 days) suggests the absence of turnover in the adult rat. However, the possibility of an appreciable re-utilization of C^{14} or a prolonged formation of cholesterol from a reservoir of labeled precursors cannot be excluded.

Summary. Incorporation of C^{14} into brain cholesterol following the intracisternal injection of acetate-1-C^{14} and pyruvate-2-C^{14} has been investigated. Incorporation was observed in both young and adult rats, but the amount of incorporation was much greater in the younger animals. The incorporation with pyruvate was less than that with acetate. Simultaneous injection of pyruvate-2-C^{14} and unlabeled acetate resulted in reduced incorporation of the C^{14} of the pyruvate. No decline in activity of cholesterol was observed during a 72 day period following the labeling of the compound by injecting labeled acetate.

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Effect of Native Dextran on Granulation Tissue Formation.* (23594)

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Lattes *et al.*(1) have suggested that acid mucopolysaccharides may be involved in stimulating the growth of granulation tissue in wounds. Wolman and Wolman(2), on the other hand, have reported that the administration of the neutral polysaccharides levan and dextran inhibited the formation of granulation tissue. We report here experiments designed to re-examine this question. Instead of using skin wounds or intraperitoneal talcum, as used by Wolman *et al.*(2), we have used the more constantly reproducible granuloma pouch(3).

Methods. Rats weighing 230 to 250 g each were used. 25 cc of air were injected subcutaneously into the backs of anesthetized animals. Into this space, 0.5 ml of a 1% solution of croton oil was immediately introduced. Two preparations of dextran were available. The high-polymer preparation (Lot No. 378, supplied by Commercial Solvents Corp.) had an average M.W. greater than 10^7. This was dissolved in 0.85% saline solution at a concentration of 40 mg/ml. The lower polymer preparation (Lot No. 359897, Commercial Solvents Corp.) had an average M.W. of 4×10^5; it was dissolved in saline solution at 60 mg/ml.

Results. In the first experiment, a daily intraperitoneal injection of 1 ml of the high-polymer dextran was administered to 9 rats and 1 ml daily of the lower-polymer solution to 10 rats. Nine control rats were not injected. On the 10th day, the animals were killed and the granuloma pouches removed.

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There were no significant differences in weight or macroscopic appearance of the pouches from the 3 groups of rats. After formalin-fixation, histological sections of the pouches were stained with hematoxylin and eosin, the periodic acid-Schiff procedure (P.A.S.), Mallory's trichrome and Laidlaw's stain for reticulin. Normal granulation tissue with good collagen formation and no evidence of infection was present in all sections. Many foam cells were seen, probably the result of the croton oil injection. No evidence of dextran deposition was found in the P.A.S.-stained sections. No differences of any sort were apparent among the 3 experimental groups.

In the second experiment, 2 groups of 10 rats each were employed. One ml of high-polymer dextran solution was injected directly into the pouches of the experimental group daily for 10 days, while 0.85% saline was similarly injected into the pouches of the control animals. Again, there were no macroscopic differences between the pouches of the 2 groups. The qualitative histological picture was similar in both groups. Normal granulation tissue with good collagen formation was found in all sections; but there was a small quantitative difference in collagen formation between the dextran-injected and control granuloma pouches. The dextran-injected pouches contained somewhat less collagen, without, however, any abnormality in orientation or staining of fibers. Histiocytes with vacuoles containing a few inclusion particles stained by the P.A.S. procedure, probably representing dextran, were noted. No evidence of infection was seen. Since the effect of highly polymerized dextran was slight, no further experiments with the lower-polymer preparation were conducted.

Discussion. These results indicate that, with the experimental conditions used, only a slight reduction in the formation of granulation tissue was achieved, even by the direct application of high-polymer dextran in high concentration. Intraperitoneal injection failed to influence granulation tissue formation in these experiments; poor absorption of the polymer from the peritoneum may account for this. Yet, Wolman and Wolman(2) reported that the growth of granulation tissue into skin incisions in rabbits was retarded following intraperitoneal injection of a very much smaller amount of levan than that of dextran used in the present experiments. In other experiments, using mice, these authors found that by injecting very large doses of levan or dextran intraperitoneally, granulation tissue formation, both in skin wounds and around talc particles in the peritoneal cavity, was inhibited.

It must be remembered when comparing the experiments of Wolman and Wolman with those reported here, that those workers used mice and rabbits instead of rats. In many of their experiments, levan was used, and the intraperitoneal dose in their series was larger than that employed in our experiments by the same route. A final difference is our use of the granuloma pouch, which, as an experimental model, is preferable to a skin wound for assessing granulation tissue formation. However, even the local application of a high concentration of native dextran resulted in only a slight diminution of granulation tissue formation, without apparent qualitative changes.

Summary. High-polymer dextran only slightly inhibited granulation tissue formation when applied directly in high concentration, but not when injected intraperitoneally in rats. No qualitative differences between the granulation tissue of experimental and control groups were observed.

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Human Milk Whey Proteins.* (23595)

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The proteins of human milk whey have received relatively little study in comparison with the readily available bovine system. From a physical-chemical standpoint this system is quite complex and numerous electrophoretic(1,2) and ultracentrifugal(2) components are in evidence. It was of interest to determine whether any of these represented or contained the blood serum albumin and γ-globulins entities. These proteins have been found in bovine milk whey(3-6) and the γ-globulins in particular have received rather extensive study.

Methods. Human milk samples collected within a week postpartum were treated with rennin at pH 4.8-5.0. The casein was removed by centrifugation and the supernatants were either *lyophilized* following dialysis against distilled water or dialyzed against a given buffer preparatory to electrophoretic, ultracentrifugal or immunochemical assay. *Electrophoretic components* were numbered in order of increasing mobility in pH 8.6, $\mu = 0.1$, sodium diethylbarbiturate buffer. Ultracentrifuge components were designated alphabetically in terms of decreasing rates of sedimentation in pH 7.4, $\mu = 0.2$ potassium phosphate buffer. Samples of the *fastest and slowest migrating components* of the milk whey were separated by pipetting of the respective ascending and descending electrophoretic boundaries following maximum resolution. These samples were used for subsequent ultracentrifugal and qualitative immunochemical experiments. Quantitative immunochemical studies employed antibodies to purified human γ_2-globulin and to serum albumin. These showed no cross-reactions which indicated that they were not contaminated with each other. The quantitative precipitin reactions were carried out as previously described(7).

Results. A typical electrophoretic pattern of a pool of 4 human wheys is shown in Fig. 1 and is in keeping with previously reported results(2). Recent studies by Schulte and Müller(1) have indicated the presence of 7 electrophoretic components 2 of which were dialyzable.

Ultracentrifuge diagrams of pooled milk whey and material obtained by pipetting of electrophoretic boundaries 1 (descending) and into area 4 (ascending) are shown in Fig. 2, the analytical values being given in Table II. The milk shows at least 5 components, the major portion consisting of a relatively heterogeneous and slowly sedimenting portion.

The proteins comprising areas 4 and 5 of the electrophoretic pattern show a major component sedimenting near 4.4 S, the residual and slower sedimenting material being relatively heterogeneous (Fig. 2). The latter proteins are analogous to the 1.6 S component of the whey and these low molecular weight materials appear to be distributed throughout the electrophoretic range of the whey. The sedimentation constant of the major component (4.4 S) indicates that it could be serum albumin(8,9). The electrophoretic mobility of the proteins in areas 4 and 5 also covers the range of albumin(10,11). Area 4 exists as a shoulder of component 3; there is a considerable amount of rather heterogeneously charged protein migrating between

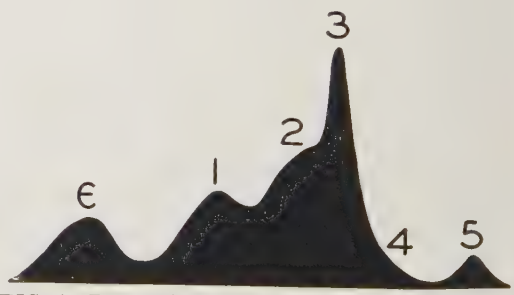

FIG. 1. Descending electrophoretic pattern of human milk whey in pH 8.6, $\mu = 0.1$ diethylbarbiturate buffer. Duration of experiment was 194 min at 4.1 volts cm^{-1}.

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TABLE I. Electrophoretic Composition of Human Milk Whey.

Sample	Component (%) 1	2	3	4	5
1	28	23	31	13	5
2	29	26	26	9	10
3	24	24	34	15	3
4	24	25	34	11	6
5	32	22	38	5	3
6	30	20	38	10	3
7	38	25	23	9	5
Range	24-38	20-26	23-38	5-15	3-10
Avg	29	24	32	10	5
Pool*	29	25	36	5	5
Avg mobilities†	2.4	3.7	4.6	5.6	7.1

* A pool of 4 samples not subjected to individual assay.

† Mobilities $\times 10^5$ cm^2 volt^{-1} sec.$^{-1}$.

areas 4 and 5.

Ultracentrifuge study of the material comprising area 1 of Fig. 1 (see lower diagram of Fig. 2) shows that the major portion consists of the above slow sedimenting material (near 1.6 S). However, 2 other components are seen. The faster of these sediments is near 17 S and is present in smaller amounts than the material sedimenting near 10.5 S. It may be analogous to the 18-19 S component of the γ_1-globulin fraction of human serum (12,13).

Immunochemical studies: Interfacial precipitin tests with the material of area 1 (Fig. 1) showed that it reacted with rabbit antibody to human serum γ_2-globulin but not with anti-human serum albumin. Attempts to demonstrate a significant amount of protein in the electrophoretic region between the salt boundary and area 1, which is the mobility region of human γ_2-globulin, were unsuccessful.

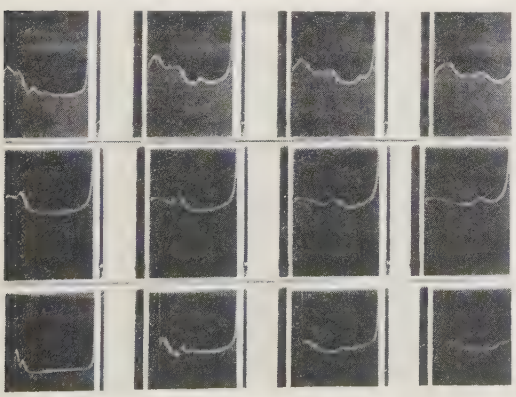

FIG. 2. Ultracentrifuge diagrams of human milk whey (upper), electrophoretic areas 4 and 5 (center) and area 1 (lower) separated by pipetting boundaries of Fig. 1. Direction of sedimentation at 59,780 r.p.m. is to the right.

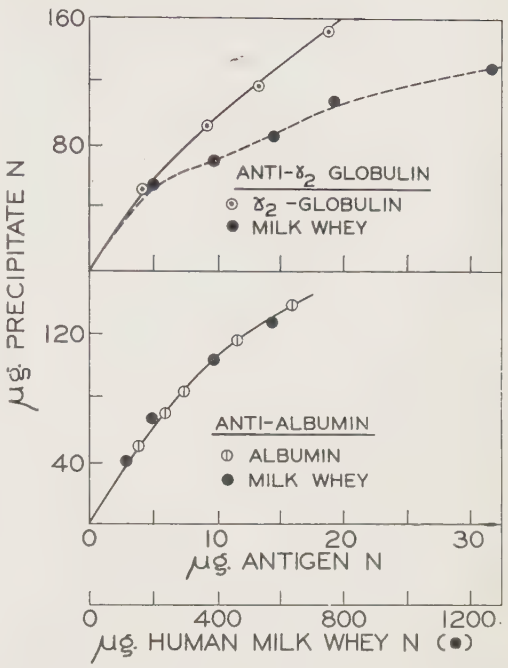

FIG. 3. Immunochemical reactions of human milk whey with a rabbit anti-human serum γ_2-globulin preparation (upper) and with a rabbit anti-human serum albumin preparation (lower).

The proteins of area 5 gave a weak reaction with rabbit antiserum albumin while those contained in areas 4 plus 5 gave a qualitatively stronger test. They did not contain components reacting with the antiserum to γ_2-globulin. The electrophoretic mobility range of these fractions would encompass serum albumin.

Quantitative precipitin studies employing pooled whey and the above antibody preparations gave the results plotted as Fig. 3. These immunochemical assays were carried out in

TABLE II. Ultracentrifugal Composition of Human Milk Whey.

Component	%	$s_{20,w}$*
a	3.0	11.7
b	6.6	10.0
c	17.5	6.0
d	17.4	4.6
e	55.5	1.6

* Values $\times 10^{13}$ cm sec.

the antibody excess region. It can be seen that the whey contains a component identical with serum albumin and that this component constitutes near 2.5% of the whey proteins The immunochemical reactions with antibody to γ_2-globulin gave assay values from an initial 2.5% to values near 1%. The data indicate that a cross-reactive protein(s) is present in the milk whey. It is known that γ_2-globulins show immunological cross-reactions with other proteins in the γ_1- and β-globulin areas(14-16) and the present results may indicate the presence of other serum proteins in the milk whey.

Summary. The results of electrophoretic, ultracentrifugal and immunochemical analyses indicate that serum albumin is present in human milk whey. The immunochemical assay shows that it comprises 2.5% of this system. Proteins with the physical properties of human γ_2-globulin could not be demonstrated although material showing immunological cross-reactions with such proteins is present.

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Inactivation of Biologically Active ("Endotoxic") Polysaccharides by Fresh Human Serum. (23596)

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Recent work in this laboratory concerned with the interaction of endotoxic polysaccharides with biological systems has utilized inactivation of T2r$^+$ coli phage as an indicator for the reaction with properdin. As expected, such polysaccharides nullified the anti-viral effect of human serum properdin. The influence of serum on other biologic activities of these polysaccharides was next investigated. The first *in vivo* phenomenon examined was the response of mouse sarcoma to a tumor-necrotizing polysaccharide after incubation with serum of known properdin content. When it was found that fresh serum abolished this property, it was first thought that this was a consequence of complexing with properdin. Contrary to expectation, however, incubation with properdin-deficient serum also inactivated the polysaccharide. These findings brought to mind unpublished experiments (1955) designed by Pillemer and Landy to detect physicochemical changes in *Salmonella typhosa* endotoxin upon exposure to fresh human serum. This treatment greatly increased the dispersion of the polysaccharide: the turbidity markedly de-

* N.I.H., Public Health Service, U. S. Depart. of Health, Education and Welfare.

TABLE I. Inactivation of Tumor-Damaging Potency of Polysaccharides with Fresh Human Serum.

Polysaccharide		Damage induced* in S37 after prior incubation of polysaccharide with:	
Source	Dose,† μg/mouse	Saline	Fresh serum
Ser. marcescens	10	9/10	0/10
S. typhosa	3	8/10	0/10
Sarcoma 37	50	10/10	2/10
Erythrocytes	40	8/10	0/10
Hellebore	30	5/10	0/10

* $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of treated mice}}$.

† Contained in 0.4 ml of saline or of fresh serum, after incubation for 1 hr at 37°C.

creased; it no longer sedimented at low speed; and it could not be centrifuged down even at high speed.† This change was also effected by RP and R3 sera. It was therefore evident to these investigators that this unsuspected attribute of serum was independent of properdin. This finding thus indicated the presence in serum of a factor, other than properdin, which is capable of modifying endotoxin. The present report deals with the biologic properties of selected polysaccharides as affected by incubation with serum.

Methods. Five polysaccharides were derived(2) from *Serratia marcescens, S. typhosa,* mouse Sarcoma 37, human erythrocytes, and a plant (Green Hellebore). Changes in potency were measured(3) chiefly with regard to induction of hemorrhagic necrosis in mouse Sarcoma 37, but also with regard to production of lethal shock in rabbits, the local Shwartzman reaction, and dermal necrosis in epinephrine-treated skin. An amount of polysaccharide, in saline, just sufficient to induce hemorrhagic necrosis in tumors was added to 0.4 ml of human serum and incubated for one hour at 37°C.

Results. Repeated experiments showed that fresh serum abolished the tumor-damaging potency of polysaccharides (Table I). However, serum heated at 56°C for 30 minutes prior to incubation, failed to inactivate the *Ser. marcescens* polysaccharide.

In the expectation that properdin might be involved, a properdin-deficient aliquot of the same human serum was prepared; to our surprise, the serum still inactivated the polysaccharide, thus indicating that properdin was not responsible for this effect. This inference was strengthened by obtaining similar results with serum completely lacking in properdin activity (prepared by addition of rabbit antiserum to human properdin). Moreover, aliquots of a serum which were made deficient, separately, in each of the components of complement and in Mg ions, all retained the ability to annul the tumor-necrotizing potency of the polysaccharides.

Lengthened incubation time increased the amount of polysaccharide counteracted by fresh serum. At 10°C no neutralization was obtained, even with incubation for 4 hours. Inactivation was the same at pH 5.8 and 7.8. KCN (1 mM) did not block the action of fresh serum, but the same concentration of $HgCl_2$ or of As_2O_3 did; as expected, cysteine (0.1 M) prevented the blocking by As_2O_3. No one of the chemical compounds, at these dose levels, damaged tumors.

In contrast to human serum, fresh serum from a rabbit failed to inactivate the polysaccharide from *Ser. marcescens.* However, serum from the same rabbit did inactivate it after the animal had been made tolerant by repeated injection of typhoid endotoxin. Fresh serum from mice had only a slight counteracting effect; that from the guinea pig was quite active. In this report human serum was used unless otherwise stated.

Continuous intravenous administration of certain polysaccharides to rabbits resulted in progressive leucopenia, hypothermia, cyanosis, respiratory difficulty and, frequently, terminal collapse(4). This sequence of "endotoxic" effects was abolished upon incubating *S. typhosa* polysaccharide with fresh serum for one hour at 37°C prior to administration by intravenous drip into rabbits. On the following day, the same animals received, by i.v. drip, an identical amount of the polysaccharide which had been incubated either with heat-inactivated serum or with

† The redistribution of endotoxin between sediment and supernatant solution was measured by 2 immunological technics in collaboration with A. G. Johnson and R-J. Trapani. Subsequently Stauch and Johnson(1) extended these observations.

saline; the typical endotoxic syndrome thereupon appeared, terminating in death. Immediate autopsy of the rabbits revealed no evidence of the renal cortical necrosis characteristic of the generalized Shwartzman reaction. If, however, the second i.v. treatment was performed with polysaccharide incubated in fresh serum, rather than in heat-inactivated serum or in saline, the endotoxic manifestations were not observed.

Fresh serum also annulled polysaccharide potency in the preparation of rabbit skin for the local Shwartzman reaction. Appropriate amounts of the various polysaccharides were incubated at 37°C for one hour with fresh serum, heat-inactivated serum, or saline. Equal amounts of these 3 reaction mixtures were injected into the abdominal skin of the same rabbits. Eighteen hours later the animals received the provocative dose of polysaccharide intravenously. The results 6 to 24 hours later showed that the fresh serum had abolished the preparatory activity of the polysaccharide whereas neither heat-inactivated serum nor saline diminished it.

It has already been shown(4) that these polysaccharides act as preparatory agents for the evocation of dermal hemorrhagic necrosis by epinephrine. After incubation for one hour at 37°C with fresh or heated serum, or with saline, polysaccharides were given i.v. to rabbits. One hour later the animals received intradermal injections of epinephrine in shaved abdominal areas. The local skin reactions were recorded after 6 and 24 hours. As was found in other phenomena, only incubation with fresh serum eliminated the effect of the polysaccharides.

Discussion. Several quite recent publications have reported on the action of fresh serum upon bacterial polysaccharides. Hegemann found that fresh human serum neutralized the pyrogenicity of crude culture filtrates from Gram negative bacteria(5); that it also neutralized purified lipopolysaccharides of these bacteria(6); and that prior heating of serum to 57°C for 45 minutes diminished its polysaccharide-neutralizing property(7). This loss of pyrogenic potency required incubation for 5 to 8 hours whereas in our experiments one hour incubation sufficed. Rowley reported that fresh rat serum split phosphate from a bacterial lipopolysaccharide (8), and that this serum activity was heat labile, appeared to require divalent ions, and exhibited a sharp pH optimum. However, no information was given on alteration of the biologic activities of the lipopolysaccharide. Ho and Kass have reported that human plasma partially protected rats against the lethal action of crude endotoxin(9), but this plasma factor was heat stable. Stauch and Johnson(1) reported on the alteration of specific precipitability of typhoid endotoxin after incubation with serum; this serum action presumably was heat stable and required incubation for 4 hours for maximum effect. Rall, Kelly and co-workers(10,11) found that fresh rabbit serum reduced, slightly, the pyrogenic activity of a bacterial polysaccharide. When rabbits were made tolerant to this polysaccharide, a greater reduction in pyrogenicity resulted. They also found that fresh rabbit serum partially protected mice against the lethal effect of a crude polysaccharide preparation, but did not protect against the tumor-necrotizing property. These diverse findings on the modifying effect of fresh serum on endotoxins refer to factors which in most instances differ from the one described in the present report, *e.g.*, one had a sharp pH maximum, and another was heat stable. Details of the present study will be reported separately.

Summary. Insofar as they have been examined, all the endotoxic properties of polysaccharides, irrespective of their source (*viz.*, bacterial, plant or mammalian), were annulled on incubation with fresh human serum but not with heated serum. Moreover, this action of fresh serum was independent of antibody, of complement, and of properdin.

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Acetylcholine-Cholinesterase Relationships in Embryonic Chick Lung Cultivated *in vitro*.* (23597)

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Acetylcholine has been shown to influence the level of cholinesterase activity of embryonic chick intestine cultured *in vitro*(1,2). Jones *et al.*(1) suggested that acetylcholine induced the formation of cholinesterase in intestinal cells, but since the added ester served only to prevent a consistent fall in cholinesterase activity during the culture period, no definite statement as to whether acetylcholine actually induced the formation of new cholinesterase could be made. The present report deals with the influence of acetylcholine and similar esters on the cholinesterase activity of embryonic chick lung, a tissue chosen because the authors hoped its normally relatively low level of cholinesterase activity would provide a setting wherein the formation of new cholinesterase following a substrate stimulus could be demonstrated. Lung cells served well for this purpose, and the system was used to study the relationship between enzyme induction and time of exposure to substrate, as well as the type of cholinesterase induced.

Methods. Primary explants of 15-day chick embryo lung were incubated at 37°C for 8 days in roller tubes in 25% embryo extract in a highly buffered salt solution according to methods described by Jones *et al.* (1). Acetylcholine and other compounds were added to the medium in amount sufficient to provide an initial concentration of 0.02 Molar. The *cholinesterase* activity and protein contents of the cells in individual roller tubes were measured by the microchemical methods described by Bonting and Featherstone(3). In experiments comparing the hydrolysis of several concentrations of various substrates, the cells of many roller tubes were pooled and homogenized, and enzyme activity was determined manometrically. Acetylcholine chloride and acetyl-β-methylcholine chloride were purchased from Merck and Company; propionylcholine iodide and butyrylcholine iodide were purchased from Dajac Laboratories at Leominster, Mass., and tributyrin was purchased from Fisher Scientific Co. Benzoylcholine was a gift from Hoffmann-LaRoche.

Results. The effects of adding 0.02 M acetylcholine or the products of its hydrolysis to the medium of embryonic chick lung cultivated *in vitro* for 8 days are shown in Table I for 2 experiments—one in which there was good growth (protein increase) of the lung cells during incubation, the other where there was no such growth. A 2 to 6-fold increase in the cholinesterase activity over the non-incubated controls occurred. If the assumption is made that cholinesterase concentration is proportional to cholinesterase activity, then these results strongly support the hypothesis that acetylcholine induced the formation of the cholinesterase. That acetyl-

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† This material is from dissertation submitted in partial fulfillment of degree Doctor of Philosophy, in the Dept. of Pharmacology, Graduate College, State University of Iowa.

TABLE I. Effects of Adding Acetylcholine (ACH) and Products of Its Hydrolysis to Medium of Embryo Chick Lung Incubated *In Vitro*.

Exp.	Treatment	No. tubes	mg protein per tube (mean)	μMoles ACH hydrolyzed/hr/mg protein (mean ± S.D.)
1 a	Not incubated	10	2.65	.58 ± .04
b	Incubated 8 days with .02 M ACH	10	4.39	3.70 ± .05*
2 a	Not incubated	6	2.10	.64 ± .09
b	Incubated 8 days with .02 M ACH	6	2.82	1.47 ± .12*
c	Incubated 8 days with .02 M choline	6	2.52	.57 ± .06
d	Incubated 8 days with .02 M Na acetate	6	2.69	.70 ± .06
e	Incubated 8 days with .02 M choline + .02 M Na acetate	5	2.07	.85 ± .04

* Significantly different from group not incubated ($p < .05$). Student's t test according to Mather(6) was used to test the significance of differences.

choline itself, and not a product of its hydrolysis, was the inducing agent was supported by the fact that sodium chloride, choline, acetate or choline plus acetate was not able to cause an increase in cholinesterase activity comparable to that produced by acetylcholine. The small increases in cholinesterase activity caused by adding choline, acetate, or both to the medium might have been due to the creation of a favorable chemical environment for the synthesis of acetylcholine by the cultured lung cells, with the acetylcholine thus synthesized acting as the inducing agent. These results are similar to those obtained with intestine(2).

The time course of the acetylcholine effect during the 8 day incubation period was studied and the results are summarized in Fig. 1, where it can be seen that the maximal increase in cholinesterase activity was reached 4 days after adding acetylcholine and was maintained only by the continued addition of acetylcholine to the medium. Enzyme activity expressed as μmoles acetylcholine hydrolyzed per hour per mg protein is the ordinate, and the abscissa is time of incubation in days. Note that incubation for 2, 4, 6 or 8 days did not change the cholinesterase level of the lung tissue, as shown by curve H. However, the addition of acetylcholine to the medium caused an increase in cholinesterase activity, as shown by curve A. There were no significant differences in the protein content of tissue incubated with or without ACH. In this particular experiment the protein content of the incubated tissue was only slightly higher than the non-incubated tissue indicating poor growth; however, the cells appeared to be multiplying well during the incubation period. Curves B, C, and D represent the fate of cholinesterase content as the result of leaving acetylcholine out of the medium from the 6th, 4th or 2nd day, respectively. Curves E, F and G show what happens if the addition of substrate to the medium is delayed until the 2nd, 4th or 6th day, respectively. When acetylcholine was added for the first 2 (Curve D) or 4 (C) days only, there remained no significant increase in enzyme activity at 8 days. When acetylcholine was added for the first 6 days only (B), the cholinesterase activity at 8 days was significantly higher than the activity of tissue incubated without acetylcholine (H),

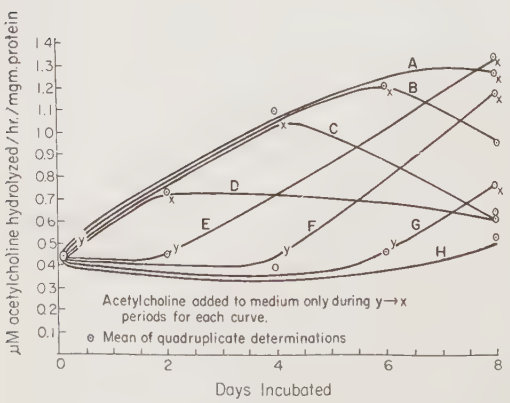

FIG. 1. Relationship of time to the effect on cholinesterase of acetylcholine added to the medium of embryonic chick lung incubated *in vitro*. Each point represents mean value of determinations from 4 roller tubes.

TABLE II. Substrate Specificity of Not-Incubated and Incubated Lung Tissue Cholinesterase.

Substrate	Substrate conc., molar	μl CO_2/hr/100 mg wet wt tissue (mean of duplicates) Not incubated	Incubated 8 days	Incubated 8 days with .02 M ACH
Acetylcholine	.1	45	50	195
	.01	55	138	428
	.001	43	83	350*
Acetyl-β-methylcholine	.1	13	73	163
	.01	13	48	118
	.001	0	13	40
Propionylcholine	.1	73	113	358
	.01	70	183	495
	.001	50	108	313*
Butyrylcholine	.1	43	1	10
	.01	41	3	6
	.001	28	0	7
Tributyrin	.1	714	126	100
	.01	352	142	94
	.001	144	92	72
Benzoylcholine†				

* Extrapolated values from 30 min. readings.
† Not hydrolyzed in any of the concentrations used.

but was also significantly lower than in the tissue incubated with acetylcholine (A) for 8 days. When acetylcholine was added for the last 4 (curve F) or last 6 (E) days only, the increase in activity produced was equivalent to that caused by adding acetylcholine for 8 days. Addition of acetylcholine for the last 2 days only (G) produced a small but statistically significant increase in cholinesterase activity.

The type of cholinesterase present in both incubated and non-incubated embryo chick lung tissue and the qualitative effect of acetylcholine were also studied. Table II shows the enzymatic hydrolysis of 6 substrates by non-incubated lung tissue. Both relative rates of hydrolysis and shape of the curves for the choline esters do not fit those of either of the 2 classical types of cholinesterase described by Augustinsson(5). Benzoylcholine was not hydrolyzed to an appreciable extent by the lung enzyme. The high enzymatic hydrolysis of tributyrin suggested the presence of a non-specific type of esterase since this substrate is reportedly not hydrolyzed by the specific acetylcholinesterase(4). On the other hand acetyl-β-methylcholine is reportedly not hydrolyzed by the non-specific cholinesterase(5); therefore, the presence of more than one type enzyme was suggested by these results.

Similar experiments were carried out with incubated tissue and the results are also summarized in Table II. After incubation for 8 days with no acetylcholine in the medium, the enzymatic hydrolysis of acetylcholine, acetyl-β-methylcholine and propionylcholine was increased while the opposite was true for tributyrin and butyrylcholine. Also after incubation an optimal substrate concentration was observed for acetylcholine and propionylcholine, which suggested the presence of an acetylcholinesterase, or "true" cholinesterase. The addition of acetylcholine to the medium increased the hydrolysis of acetylcholine, acetyl-β-methylcholine and propionylcholine and did not affect the hydrolysis of tributyrin and butyrylcholine.

Since the data on the substrate specificity of the enzyme in the incubated tissue indicated that there were at least 2 enzymes present, a suggested explanation of the results would be the following: The original non-incubated lung tissue contained both an acetylcholinesterase and a non-specific esterase capable of hydrolyzing certain choline esters. This non-specific esterase was more specific for aliphatic esters, as evidenced by

the high hydrolysis rates of tributyrin and butyrylcholine compared to the low rate for benzoylcholine, and exhibited maximal hydrolyzing powers at high substrate concentrations. When the lung was incubated *in vitro* for 8 days, the acetylcholinesterase activity increased with the growth of the cells and the non-specific esterase activity decreased. Thus, the acetylcholinesterase activity would tend to become "unmasked," explaining the optimal substrate concentrations observed for acetylcholine and propionylcholine. Addition of acetylcholine to the medium increased the activity (concentration) of acetylcholinesterase only, explaining why the enzymatic hydrolysis of tributyrin and butyrylcholine was not affected. This suggested explanation is compatable with the data and indicates that, at least under the experimental conditions of the reported study, a physiological substrate of an enzyme has a role in the formation of that enzyme.

Summary. Addition of 0.02 Molar acetylcholine to the medium of 15-day embryo chick lung cultivated *in vitro* for 8 days caused a 2- to 6-fold increase in the cholinesterase activity of that tissue. The increase in enzyme activity was maximal 4 days after addition of acetylcholine to the medium, and was maintained only by the continued addition of acetylcholine to the medium. Neither choline nor acetate alone nor choline and acetate together produced an increase in enzyme activity comparable to that produced by acetylcholine. Results of substrate specificity studies on the cholinesterases of chick embryo lung suggested that there was both an acetylcholinesterase and a non-specific esterase in the lung cells. Only the acetylcholinesterase was affected by the added acetylcholine. The results indicated that acetylcholine was an inducing agent for the formation of acetylcholinesterase in the embryo chick lung cells cultivated *in vitro*.

Thanks are due to Miss Patricia Sandy and William Coffey for their technical assistance.

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Relationship Between Penicillin Susceptibility and Virulence of *Staphylococcus aureus.* (23598)

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Penicillin susceptibility and virulence as measured by intraperitoneal mouse pathogenicity with mucin(1) of 80 recently isolated strains of staphylococci were determined and compared to ascertain whether or not any relationship exists between both characteristics. A good degree of reproducibility for a biological procedure has been observed with the animal virulence test for staphylococcal pathogenicity provided young Swiss mice and a constant, moderate-sized inoculum of microorganisms are employed. To further elucidate this problem, 4 penicillin-sensitive staphylococcal strains were assessed for virulence before and after induction of penicillin resistance *in vitro.* Since repeated artificial passage on media containing graded concentrations of penicillin might conceivably result in loss of virulence by itself, each strain was also transferred on antibiotic-free media an equal number of times as that required to induce resistance and also tested for virulence.

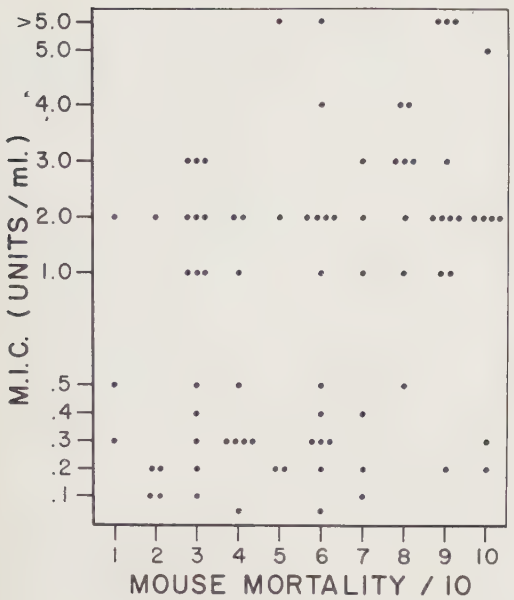

FIG. 1. Comparison between penicillin susceptibility and intraperitoneal mouse pathogenicity with mucin of 80 coagulase positive, hemolytic, mannitol fermenting strains of *Staphylococcus aureus*.

To establish a uniform basis for comparison, only coagulase positive, hemolytic, distinctly pigmented, mannitol fermenting strains were employed.

Methods. The tube dilution method was used to determine penicillin sensitivity. Medium was fresh veal extract broth, pH 7.2-7.4. Test was performed in 2 ml amounts, by adding 1 ml of a 10^{-6} dilution of a 6 hour broth culture of the test strain to a series of tubes containing varying concentrations of standard penicillin in 1 ml of broth. The minimal inhibitory concentration is defined as the smallest amount of antibiotic which completely inhibited growth after 18 hours of incubation at 37°C. A growth control tube containing the test inoculum without antibiotic was used with each determination. Ten albino Swiss mice, weighing approximately 15-20 g, were used to assess the virulence of each strain. 0.5 ml of 5% hog gastric mucin, prepared according to Miller(2), was injected intraperitoneally into each mouse followed within 15-20 minutes by 0.25 ml of a 16-20 hour fresh veal extract broth culture of the strain under test adjusted to an optical density of 0.05 on a Photoelectric Lumetron colorimeter, using a red filter (650 mμ) and which contained approximately 150 million organisms. Animals were observed 48 hours and deaths recorded. Penicillin resistance was induced by serial subculture of each strain on agar plates containing progressively increasing concentrations of antibiotic. Inoculum for each passage was obtained from preceding growth on plates containing the highest concentration of penicillin. Each strain was also serially transferred on antibiotic-free agar slants an equal number of times as that required to induce resistance.

Results. Mortality in mice produced by each strain following intraperitoneal injection with mucin in relation to its penicillin sensitivity is shown in Fig. 1.

Considerable dispersion of mouse pathogenicity may be observed among strains of the same antibiotic susceptibility. Although no distinct correlation is found between both factors, a greater concentration of strains producing a higher mortality is noted among those requiring larger amounts of penicillin to effect growth inhibition as compared to those strains inhibited by smaller amounts where mouse mortality is fairly evenly distributed throughout. The status of all 80 strains with regard to antibiotic sensitivity and virulence is summarized in Table I. As may be noted from the table, the 42 penicillin-sensitive strains were almost evenly divided between those eliciting a mortality of up to 50% (5/10) and over 50% (6/10), 23 and 19 respectively. On the other hand, of the 38 antibiotic-resistant strains only 11 or 28.9% proved avirulent by the above criterion while 27 or 71.1% were virulent. When these data

TABLE I. Mouse Mortality of Staphylococcal Strains in Relation to Their Penicillin Susceptibility.

Penicillin susceptibility	Total No. strains	Mouse mortality: Up to 50% No.	%	Greater than 50% No.	%
Sensitive*	42	23	54.8	19	45.2
Resistant	38	11	28.9	27	71.1

* Strains inhibited by 1.0 unit of penicillin/ml or less are classified as sensitive, and those requiring larger amounts as resistant.

TABLE II. Intraperitoneal Mouse Pathogenicity with Gastric Mucin of Penicillin-Sensitive Strains of *Staphylococcus aureus* before and after Artificial Induction of Resistance to Penicillin.

Strain	Mouse mortality/10		
	Original isolate	Passage control on antibiotic-free medium	After induction of penicillin resistance
25	2	1	0
88	2	4	1
89	4	4	9
90	7	4	0

were subjected to statistical analysis by means of the chi square test,* a chi square of 5.44 and a P value of 0.02 was obtained thereby indicating that the observed differences were definitely significant. Thus based upon the above findings with the series of staphylococci investigated it may be concluded that although no direct relationship is evident between penicillin susceptibility and virulence, penicillin-resistant strains are more apt to be virulent than are those sensitive to the antibiotic.

Mouse pathogenicity of 4 penicillin-sensitive strains before and after induction of resistance as well as the mortality produced after passage on antibiotic-free agar is recorded in Table II.

After serial transfer on graded concentrations of antibiotic, strains #25, 88, 89, and 90 were able to grow on agar plates containing 80, 100, 1,000, and 400 units of penicillin per ml whereas their original isolates as well as their subcultures on antibiotic-free media were inhibited by 0.05, 0.1, 0.5, and 0.05 unit of penicillin per ml respectively. No consistent pattern with respect to change in virulence following induction resistance is discernible. Thus, strains #25 and 88 exhibited a slight decrease, strain #89 a marked increase, and strain #90 a marked decrease in virulence after being made resistant.

Summary. No distinct correlation was established between penicillin susceptibility and virulence of 80 coagulase positive, hemolytic, mannitol fermenting strains of *Staphylococcus aureus.* Both penicillin-resistant and penicillin-sensitive strains vary considerably in their mouse pathogenicity but a significantly higher proportion of the former proved to be virulent as compared to the latter which were fairly evenly divided between virulent and avirulent strains. No definite pattern with respect to change in virulence was observed with 4 originally penicillin-sensitive strains following artificial induction of resistance to the antibiotic.

* We are greatly indebted to Mrs. Constance Percy for the statistical analysis.

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Establishment of Human Adult Tonsil Cells in Continuous Culture and Their Virus Susceptibilities.* (23599)

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This paper describes the development in tissue culture of a continual line of epithelial-like cells from adult human tonsil tissue and compares the susceptibility of these cells to a number of viruses with that of a human carcinoma cell line (strain HeLa). The tonsil line has been subcultured serially over 40 times in the course of a year and has been maintained from the start in a medium free from human serum.

* Supported in part by contract between Office of Naval Research and the University of Wisconsin and a grant (E1299) from the Nat. Inst. of Allergy and Infectious Diseases.

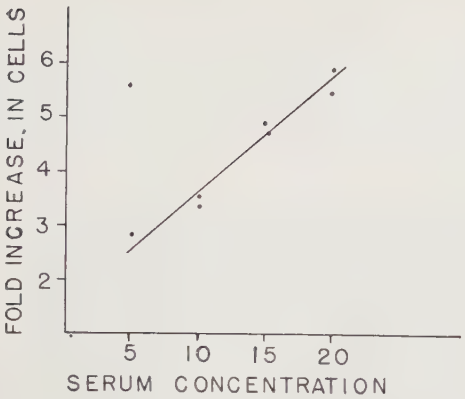


FIG. 1. Effect of varying % of serum concentration on fold increase in tonsil cells after 4 days. Medium 199 was used throughout.

Materials and methods. Tissue culture technics were similar to those generally employed(1). Mixture 199(2) was used for growth of cells with 10% calf serum (Cappel). For maintenance of cells, Scherer's solution(3) with 5% calf serum was satisfactory. In the initial growth of tissue fragments, minced pieces were cultivated using a plasma clot technic. In each ml of media 100 units of penicillin, 100 μg of streptomycin and 50 units of nystatin were routinely incorporated. The viruses employed included adenovirus types 1-7, some obtained through Dr. Robert Huebner, poliomyelitis virus types I-III representing standard prototypes from Dr. Gilbert Chang, mumps virus (Enders' strain), Coxsackie virus strains from Dr. Lois Kitze, hemagglutinating virus of Japan (Sendai strain) from Dr. D. L. Walker, Newcastle disease virus of the Victorian and G.B. types from Dr. R. L. Hanson, influenza strains from the Naval Medical Research Unit at Great Lakes, vaccinia virus from Lederle calf vaccine, and an intranuclear inclusion-producing virus resembling measles isolated in this laboratory from monkey kidney cells. Titrations were made by inoculation of half log dilutions into 4 tubes for each dilution and are expressed as log TC 50/ml of inoculum. The final reading was made on different days depending on the virus tested.

Results. Clinical data: Tonsil designated as T16 was obtained from a 24-year-old female student nurse who had had enlarged tonsils for 15 months and repeated sore throats. Surgery was performed on January 31, 1956 under local anesthesia.

Establishment of cell line: Tubes of tonsil tissue were prepared by a plasma clot technic and some were maintained in the original tubes for 9 months. No cytopathogenic effect was noted and no virus could be demonstrated in the supernatant fluid tested over 11 times from the 11th to 190th day of culture using

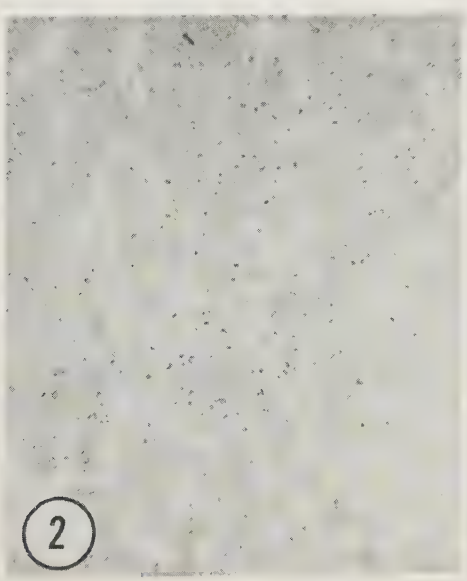
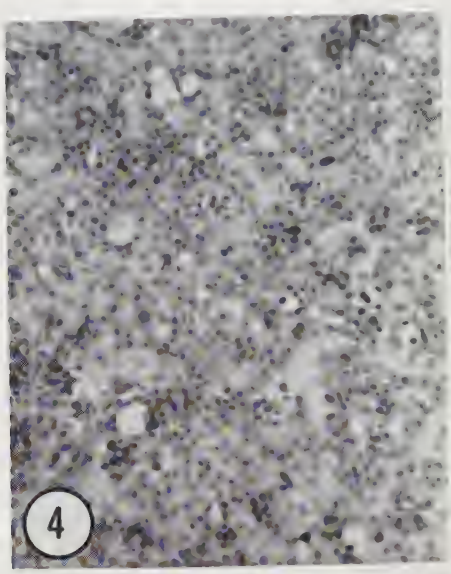
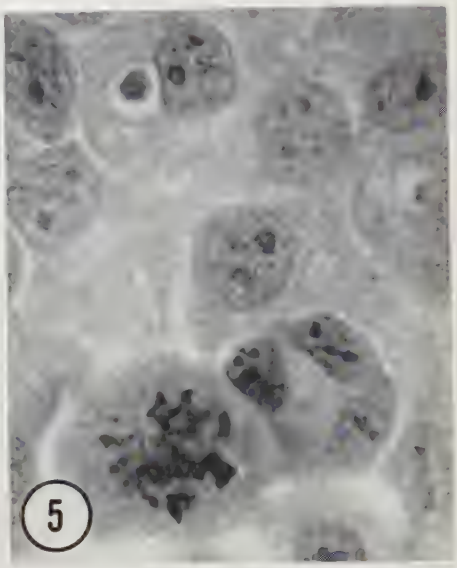


FIG. 2. Unstained preparation of the tonsil line showing cell sheet. × 87.
FIG. 3. Phase microscope appearance of heavy sheet of tonsil cells. × 87.

HeLa, human intestine, human amnion, human tonsil, and monkey kidney cells. No complement fixing activity for adenovirus was found in the supernatant fluid. Repeated histological sections stained with hematoxylin and eosin failed to reveal foamy changes, intranuclear inclusions, or other evidence suggesting a virus infection. Initial growth was both epithelial and fibroblast in appearance. After 2 months cultivation, epithelial-like material from 2 of the original tubes was scraped off, pooled, and implanted in a serum





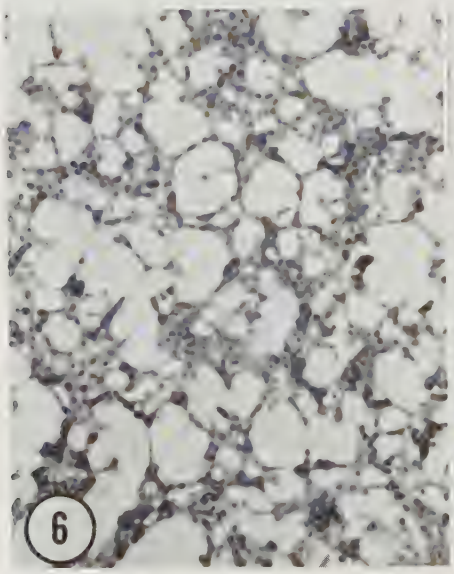


FIG. 4. Collodion preparation of tonsil cells in 19th subculture. Hematoxylin and eosin stain. × 87.

FIG. 5. Same at higher magnification showing mitotic figure, small aggregate of nuclei, and variation in nucleoli. × 667.

FIG. 6. Stained preparation of tonsil cells 8 days after inoculation with adenovirus type 7 showing disruption of cell sheet. Hematoxylin and eosin stain. × 87.

FIG. 7. Same under higher power showing nuclear changes. × 667.

sampling bottle. After 3 such passages 0.5% trypsin was used for 5-10 minutes at room temperature to disperse the cells. Over 40 subcultures have been made over a period of a year each in the complete absence of human serum.

Growth characteristics: Total cell counts on tonsil cells exposed to trypsin were made at different intervals to determine the best growth medium. At a 10% serum concentration Mixture 199(2), the maintenance-tryptose phosphate combination described by Ginsberg(4), and the basal medium of Eagle's for HeLa cells(5) all resulted in about a 4-fold increase in cells over a week's period. Scherer's maintenance medium(3) resulted in no appreciable multiplication under similar circumstances. *Serum concentration* influenced the growth of cells. This is shown in Fig. 1 which records the results of duplicate counts made on cells grown in bottles for a 4 day period. An almost linear increase in cell population was found as serum concentration increased. *Scherer's maintenance* with 5% calf or horse serum maintained cells well for 2 and sometimes 3 weeks with changes at 3-4 day intervals. A maintenance medium of Hanks balanced salt solution (94.5 parts), lactalbumin hydrolystate (0.5 parts) and a calf serum (5 parts) could be used for short term experiments of less than a week's duration. The cells could be stored without degeneration in a 30°C incubator for 2-3 weeks prior to inoculation.

Morphology: In unstained preparations the tonsil line was epithelial-like in appearance and yielded a solid sheet of cells when grown on glass with appearance of a few rounded cells after maintenance of a week or more (Fig. 2-3). They could not be differentiated readily from other cell lines originating from normal human tissues (intestine, liver, kidney, conjunctiva) or malignant human tissues (KB, Hep-2, HeLa) maintained in this laboratory. Stained preparations showed epithelial-like cells and occasionally darker stained and somewhat smaller cells resembling lymphocytes (Fig. 4-5). Alterations in both the stained and unstained cell preparations were seen after exposure to different viruses. The late effects of adenovirus (type 7) are illustrated in Fig. 6-7. Nuclear changes in stained cells were also observed with poliomyelitis virus and with an agent resembling measles which produced a syncytial pattern, multinucleated giant cells, and eosinophilic intranuclear inclusion bodies.

Virus effects: A number of viruses have been qualitatively tested for their capacity to produce cytopathogenic changes in tonsil epithelium (T16) with simultaneous testing in a strain of HeLa cells adapted to growth in horse serum. The results are presented in Table I. The tonsil line is susceptible to a wide range of viruses similar to the HeLa line. In addition, the tonsil line has been successfully employed in the original isolation of adenoviruses, herpes simplex virus, and mumps virus. It has been used as a source of adenovirus antigen for complement fixation tests and in neutralization tests for adenovirus, herpes simplex, and poliomyelitis. Hydrogen ion changes permit color tests with adenovirus.

Virus titrations: Susceptibility of the tonsil cell line was compared to that of the HeLa line using several viruses. The growth, maintenance media, and inocula were the same for both cell lines and the titrations were carried out simultaneously. The results are presented in Table II. High titers in both HeLa and tonsil cells (T16) were found for adenovirus, Coxsackie, and poliomyelitis viruses with cytopathogenic changes that were early in appearance (except for adenovirus 4) and clear cut in effect. No real difference was found between the 2 cell lines.

Discussion. The feasibility of establishing a line of cells in continuous culture from normal adult human tonsil tissue has been demonstrated. The line described here is a vigorous growing, relatively hardy one carried from the start in the absence of human serum. It has a wide range of virus susceptibilities similar qualitatively and quantitatively to the HeLa cell lines. While this line has been free from latent virus infection, it should be recalled that adenovirus and salivary gland virus may appear in some "normal" tonsil or adenoid tissue after prolonged cultivation (6-7) in a combined frequency in a younger

TABLE I. Viral Susceptibilities of Tonsil Cells (T16) and HeLa Cells.

Virus group	Strain or type	Virus source	Single dilution tested*	Cytopathogenicity Tonsil (T16)	Cytopathogenicity HeLa
Adenovirus	1	HeLa culture	10^{-4}	+	+
	2	"	"	+	+
	3	"	"	+	+
	4	"	"	+	+
	5	"	"	+	+
	6	"	"	+	+
	7	"	"	+	+
	Bertha	"	10^{-1}	+	+
Coxsackie	A2 (Dalldorf 2)	Suckling mouse	10^{-3}	0	0
	3 (" 3)	"	"	0	0
	B1 (Conn 5)	"	"	+	+
	2 (Nancy)	"	"	+	+
	2 (Ohio)	"	"	+	N.D.
	E4	"	"	0	0
	High Point	"	"	0	0
	Wiederhold "C"	"	"	0	0
Hemagg. virus of Japan	Sendai	Allantoic fluid	"	0	0
Herpes simplex	H.F.	HeLa culture	"	+	+
Influenza	A_1 (PR^8)	Allantoic fluid	10^{-4}	0	0
	A (Great Lakes)	"	"	0	0
	B (Lee)	"	"	0	0
Measles	Wisconsin	"	Undil.	0	0
Mumps	Enders'	Amniotic fluid	10^{-2}	+	+
Newcastle	Victorian	Allantoic fluid	10^{-4}	+	+
	G.B.	"	"	+	+
Poliomyelitis	I (Mahoney)	HeLa culture	"	+	+
	II (Lansing)	"	"	+	+
	III (Leon)	"	"	+	+
Vaccinia	Lederle	Calf vaccine	"	+	+

* Indicates dilution of original material inoculated into tissue culture tubes. Only one dilution for each virus was tested and 0.1 ml inoculum was used.
N.D. = Not done.

age group approximating 55%. This is of course a great disadvantage in establishing continuous cultures from such cells. There should be an observation and testing period of about 2 months to eliminate this possibility.

Two other similar epithelial lines of tonsil cells have been established in this laboratory. One was deliberately discontinued after several trypsinized subcultures and the other is in the 9th passage. Fibroblast-like lines have also been started with both human tonsil and human lung but subcultured only with difficulty.

TABLE II. Comparative Titrations of Certain Viruses in Tonsil (T16) and HeLa Cells.

	Type	Day of reading	Virus titer* Tonsil	Virus titer* HeLa
Adenovirus	2	6	5.25	5.25
	3	6	4.75	4.75
	4	7	5.75	5.75
Coxsackie	B1 (Conn 5)	3	7.25+	7.25+
	B2 (Nancy)	3	7.25+	7.00+
Polio	I (Mahoney)	6	7.75	7.75
	II (Lansing)	6	9.50+	8.75+
	III (Leon)	6	9.50+	9.50+

* Log TC 50/ml of inoculum.

Summary. 1) A line of epithelial-like cells derived from tonsil tissue surgically removed from a normal adult female has been established in serial culture in the absence of human serum and maintained for over 40 passages over the course of a year. 2) Cytopathogenic effects in this line of tonsil cells were produced by 7 types of adenoviruses, 2 Coxsackie virus strains, 3 poliomyelitis prototype viruses, 2 Newcastle Disease virus strains, herpes simplex virus, mumps virus, vaccinia virus, and after adaptation, an intra-

nuclear inclusion-producing agent resembling measles virus. No clear cut effect was seen with 6 other Coxsackie strains, 3 influenza prototype viruses, or hemagglutinating virus of Japan. 3) Simultaneous titration of several adenovirus, poliomyelitis, and Coxsackie virus strains in tonsil and HeLa cells gave high and comparable titers.

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On Inhibition of Vit. K Activity by Sulfaquinoxaline in Chicks.*† (23600)

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Supplementation of poultry rations with sulfaquinoxaline is known to increase the requirement of chicks for vit. K(1,2). Mushett and Seeler(3) have shown that the action of this sulfonamide is not likely to be related to curtailment of the biosynthesis of vit. K in the intestinal tract, or to histological changes in the liver. A quantitative relationship between sulfaquinoxaline and vit. K has been indicated by preliminary work done at the Ill. Agric. Exp. Station. It is the purpose of the present report to investigate some aspects of this relationship.

Methods. Female crossbred chicks were kept on a purified diet deficient in vit. K to the age of 2 weeks, when the chicks were distributed into groups of 10, and graded levels of sulfaquinoxaline (0.025—0.4%) added to their diet. The basal diet is given in Table I. After 6 days graded levels of menadione sodium bisulfite complex (MSBC) dissolved in water, and containing 33% 2-methyl-1,4-naphthoquinone, were force-fed to the chicks. As the feed, and therefore the intake of sulfaquinoxaline, were influenced by the size of the chick, the doses of MSBC were adjusted to the body weight of each chick and were expressed in μg/100 g of body weight. Twenty-four hours later blood was drawn by heart puncture and plasma prothrombin times determined by a modification of the onestage method of Quick(4), using sodium citrate as an anticoagulant and acetone-dehydrated chick brain as a source of thromboplastin.§ Due to considerable

TABLE I. Composition of Basal Diet.

Ingredient	%
Drackett protein	30
Cerelose	30
Starch	29.36
Salt mixture*	5.34
Solka floc†	3
Refined corn oil	1
Glycine	.50
DL-methionine	.40
Choline-Cl	.20
Total‡	100

* Griminger *et al.*(6).

† Non-nutritive fiber, containing 99.5% cellulose.

‡ Plus the following vitamins (mg/kg diet): Thiamine HCl 25; riboflavin 16; niacin 100; calcium pantothenate 20; pyridoxine HCl 6; folic acid 4; biotin 0.6; cyanocobalamine 0.02; ascorbic acid 200; alpha-tocopherol acetate 20; 10,000 I.U. vit. A and 600 I.U. vit. D_3.

§ An outline of the method employed will be sent on request.

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† This work was supported in part by a grant from Abbott Laboratories, North Chicago, Ill.

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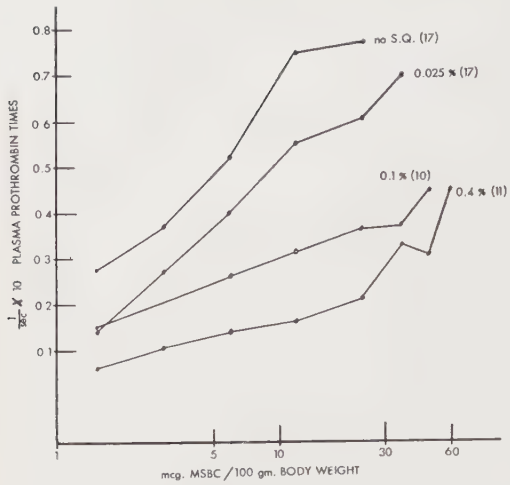

FIG. 1. Effect of single doses of menadione sodium bisulfite complex (MSBC) on plasma prothrombin times of chicks fed various levels of sulfaquinoxaline. Figures in parentheses refer to number of chicks on each ration finishing the experiment.

mortality in the lots getting higher levels of sulfaquinoxaline, the experiment was repeated with 12 chicks/lot. Fig. 1 shows the averages of all the prothrombin values obtained for 4 levels of the drug with different doses of MSBC. Average prothrombin times of the chicks not receiving doses of MSBC were 116 sec. without sulfaquinoxaline, 154 sec. for the 0.025% level, and over 180 sec. for the 0.1 and the 0.4% levels of the drug. These control values were not included in the log-dose-response presentation (Fig. 1).

In another experiment, 3-week-old K-deficient chicks were given a single dose of 2 μg menadione, dissolved in refined corn oil, /100 g body weight. Half of the group of 36 chicks were left on the control diet, the other half were given the diet supplemented with 0.2% sulfaquinoxaline. Four chicks were taken from each group every 24 hours for plasma prothrombin time determinations. The results of this trial are shown in Table II.

TABLE II. Effect of Sulfaquinoxaline on Prothrombin Times of Chicks after Receiving 2 μg Menadione/100 g Body Weight.

Days after menadione dose	Avg plasma prothrombin times in sec.	
	Control diet	0.2% sulfaquinoxaline diet
1*	17 (578 ± 35)†	20 (502 ± 19)
2	24 (411 ± 59)	40 (251 ± 11)
3	37 (271 ± 45)	102 (98 ± 18)
4	49 (206 ± 18)	180 (56)‡
5	58 (172 ± 11)	

* Four chicks from each group bled each day.

† Figures in parentheses represent reciprocal prothrombin time avg and stand. errors both $\times 10^4$. $\sqrt{\frac{(\Sigma d^2)}{n(n-1)}}$,

‡ None of the samples clotted within 180 sec.

TABLE III. Influence of Single Doses of Sulfaquinoxaline and Menadione on Prothrombin Times.

Sulfaquinoxaline	μg menadione/mg sulfaquinoxaline	Individ. plasma prothrombin times in sec.	Avg plasma prothrombin times, sec.*
—	—	40, 90	56
+	—	115, 119, 180+	132
+	.1	19, 28, 32	25
+	.2	19, 21, 24	21
+	.6	14, 15, 17	15
+	1.0	13, 13, 17	14
+	2.0	13, 14, 16	14

* Mean values on the basis of reciprocals of individual plasma prothrombin times.

In a third experiment each of a group of 20 chicks, selected for similar body weight (mean weight = 269 g, std. error = 1.6 g) received a dose of approximately 50 mg sulfaquinoxaline in a gelatine capsule (range 46.8-56.1 mg); the capsule was followed within 20 minutes with graded doses of menadione, dissolved in refined corn oil (10 μg/ml), except for a number of controls. The levels of menadione that were fed were defined in μg/mg of sulfaquinoxaline given to each individual chick. Plasma prothrombin times were determined after 24 hours. The results are given in Table III.

In all trials, the chicks were raised in electrically heated batteries equipped with thermostatic temperature controls and raised wire floors. Feed and water were kept before the birds at all times; to minimize bacterial synthesis of vit. K, the water was changed daily and the containers cleaned carefully.

Prothrombin times were averaged on the basis of the reciprocals of the individual clotting times(5); the averages were then reconverted into seconds. Observations were discontinued if no clot was observed after 180 seconds, and "180" used in the calculations. The use of reciprocals helps to mini-

mize inaccuracies in this range.

Results. Fig. 1 indicates that when higher levels of sulfaquinoxaline are incorporated into the feed, larger single doses of MSBC have to be supplied to obtain a given level of prothrombin. Although it might be an empirical relation, it has been shown that over the sensitive range a plot of the mean reciprocal prothrombin times against the logarithm of the vit. K dosage yields practically a straight line(5). The data presented in Fig. 1 seem to approach such lines, within the normal range of error, for each of the levels of sulfaquinoxaline in the feed. There appears to be a tendency for a decrease in slope with increasing sulfaquinoxaline levels. If this decrease is real, it would mean that with higher levels of the inhibiting compound, more of the vitamin per unit of inhibitor is required to overcome the inhibition. Experiments to investigate this proposition are presently under consideration.

The role of vit. K in the synthesis of prothrombin has not been clarified. Therefore, hypotheses about the mode and type of inhibition of this synthesis must, for the present time, remain in the realm of speculation. So far it can only be stated that the oral administration of sulfaquinoxaline will inhibit the formation of prothrombin, as measured by the one-stage method, that this inhibition can be reversed by the administration of vit. K-active compounds over a wide range, and that there appears to be a relationship between the quantities of inhibitor and of vitamin that can overcome the inhibition.

A single dose of 2 μg of menadione/100 g body weight, given to vit. K-deficient chicks, will lower prothrombin times appreciably, without, however, increasing prothrombin levels to normal. Decreases in prothrombin, as manifested by increased prothrombin times, were observed in Exp. 2 from day to day. After 5 days prothrombin times were approximately as long as before the dose of menadione had been given. The "residual" level of prothrombin found at that time was probably due to amounts of vit. K in the feed ingredients, or to intestinal synthesis, or both, and was probably less than 5% of the normal level. When 0.2% sulfaquinoxaline was included in the ration, this level of depletion was reached in half the time, and nearly complete depletion was achieved within 4 days, as shown in Table II.

An attempt was made in Exp. 3 to estimate the amount of menadione necessary to overcome a certain amount of sulfaquinoxaline, when both were given in the form of a single dose to vit. K-deficient chicks. With chicks of this age, the individual dose of 50 mg corresponds approximately to the daily intake of chicks receiving 0.15% of sulfaquinoxaline in their ration. The prothrombin times of each of the 20 chicks involved in this test, as measured after 24 hours, are given in Table III. Trial 1 of this series, and other experiments have shown that for depleted chicks of this weight, and in the absence of inhibitors, a single dose of 16-22 μg of menadione will suffice to restore normal prothrombin times. Although it appears that there is a certain amount of individual variation in the sensitivity of chicks to sulfaquinoxaline, an overall ratio of menadione to sulfaquinoxaline can be estimated for this range of the drug used. From the data shown in Table III it can be calculated that in the range studied, and after subtracting the menadione required for normal prothrombin levels in the absence of inhibitor, about 1.2 μg of menadione were required to overcome the inhibitory effect of 2 mg of sulfaquinoxaline. As the drug has about twice the molecular weight of menadione, over 800 moles of sulfaquinoxaline were required in this trial to inhibit the prothrombin-forming activity of 1 mole of menadione. It has to be stressed that these data were calculated on the basis of the amount of vitamin and drug ingested; rate and degree of absorption could change materially the ratio of the compounds interacting at the site of prothrombin formation.

Conclusion. Increasing doses of vit. K-active compounds are necessary to overcome the inhibition of prothrombin formation in chicks fed increasing levels of sulfaquinoxaline. The drug will hasten depletion of prothrombin in chicks kept on a K-deficient ration. When a single dose of about 50 mg

of sulfaquinoxaline was administered orally with varying levels of menadione, one mole of menadione appeared to overcome the inhibitory effect of more than 800 moles of sulfaquinoxaline.

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Non-Transfer of *Trans* Fatty Acids from Mother to Young.*† (23601)

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Bertram(1) first reported the presence of traces of *trans* Δ11 octadecenoic acid in oxen, sheep and butterfat. More recently Swern *et al.*(2) reported that beef fat contained not only traces but between 5 and 10% *trans* fatty acids. Hartman and his coworkers extended these investigations to the depot fats of various animals(3) and reported that whereas ruminants contained considerable amounts of *trans* fatty acids, non-ruminants and birds contained little or none. Reiser(4) has suggested that the occurrence of *trans* fatty acids in ruminants is due to the activity of the intestinal flora. Although *trans* fatty acids do not normally appear in the depot fats of non-ruminants *trans* fatty acids are deposited in the tissues whenever they are fed as a dietary component(5-7). It therefore, appeared of interest to note whether *trans* fatty acids were transferred to the tissues of non-ruminants by other than dietary means.

In the present study considerable amounts of *trans* fatty acids were deposited in the tissues of female rats by feeding these acids in the form of hydrogenated margarine stock. The rats were then mated and both mother and young analyzed for their *trans* fatty acid content.

Methods. One kg of the basal diet was prepared from glucose 640 g, casein 310 g, and Wesson salt mix(8) 50 g. Four g of a water soluble vitamin mix was added to each kg of feed(9) and the fat soluble vitamins were given by dropper twice each week.‡ Fifteen % of hydrogenated margarine stock containing 40.7% *trans* fatty acids was added to the feed at the expense of the glucose. Two % of corn oil was also added at the expense of glucose to serve as a source of essential fatty acids. Three rats were sacrificed at the beginning of the experiment, and 10 rats were placed on the experimental diet for 73 days and then mated. Eight mothers and their litters were sacrificed by anesthetisation with ether immediately after birth of the young. The remaining 2 litters were allowed to suckle the maternal milk for 9

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‡ One drop/rat of the following vitamin mixture administered twice each week. Five g vit. A (200,000 U.S.P. units, courtesy Distillation Products), .0054 g vit. D_2 and 2.535 g vit. E (mixed tocopherols) in 100 ml of olive oil.

TABLE I. Percentage of *Trans* Fatty Acids Passed on by a Mother Rat to Her Young.

No.	Mother		Young		No. of young in litter†	No. of young born alive
	Total carcass fat, g	*Trans* fatty acid in carcass fat, %	Total carcass fat, g	*Trans* fatty acid in carcass fat, %		
1	41.8	23.5	.68	<.5	12	9
2	38.8	26.2	.76	.5	12	10
3	38.6	26.8	.55	.5	10	9
4	37.5	25.1	.42	.5	9	9
5	43.2	24.0	.82	.5	12	11
6	40.8	26.6	.32	.5	7	7
7	37.6	26.6	.75	.5	11	11
8	38.6	25.7	.40	.5	10	9
9	27.9*	26.1	3.3	24.3	9	8
10	20.1*	23.8	3.3	24.8	10	8

* Last 2 litters allowed to suckle 9 days.

† This number represents the number of young analyzed except for No. 9 and 10 in which cases only those rats surviving suckling period were analyzed. These numbers were 7 and 6 respectively.

days before the young were sacrificed. The fat was extracted from the carcasses by a method previously described(9). The percentage of *trans* fatty acid was determined by use of a Beckman IR2A infra red spectrophotometer and the Jackson and Callen baseline method(10). All the analyses were done in carbon disulfide solution at a 5% concentration and the results based on a trielaidin standard.

Results. Although the carcass fat of female rats which had been fed margarine stock contained between 23.5-26.8% of *trans* fatty acids, less than 0.5% of *trans* fatty acids was found in the carcass fats of their young at birth (Table I). On the other hand, the carcass fat of the young which were allowed to suckle the maternal milk for 9 days contained approximately the same percentage of *trans* fatty acids as their mothers.

No *trans* fatty acids have been detected in adult rats unless they had received a dietary source of *trans* fatty acids(9). Fats extracted from rats fed olive oil have been shown to contain only traces of absorption at 10.3 μ. This absorption represented less than 0.5% of *trans* fatty acid and may have been due to the limitation of the method of analysis or may have been due to the transfer of *trans* fatty acids from the breeding colony§ during the weanling period. The amount of total fat extracted from young which were allowed to suckle increased substantially, as is normally the case in suckling rats(11). It was observed that the yield of total fat from the mothers of these young was lower than that of the mothers sacrificed on the birth of their young (Table I). It would thus appear that the mothers mobilized considerable quantities of their depot fat, some of which was used in the production of milk fat. A marked increase in the *trans* fatty acid content of young therefore resulted.

The observation that the *trans* fatty acid content of the young increased on suckling is in agreement with a study by McConnell and Sinclair(12). They also found a large increase in the *trans* fatty acid content in the fat extracted from rats which had suckled the milk of a mother fed a diet rich in elaidic acid. However, these authors reported the finding of 10% *trans* fatty acids in the depot fat of the young immediately after birth. The present study does not confirm this finding. There are 2 possible explanations for this conflict. As has been previously pointed out, the method of analysis used by these workers has recently been shown to be unreliable(10). Furthermore, the rats used in the study by McConnell and Sinclair received a much higher quantity of dietary *trans* fatty acids than the rats in the present study. The passage of considerable quantities of *trans* fatty acids to the young may have been a result of overloading the mother rat with *trans* fatty acids.

The number of young in each litter and

§ Holtzman Co., Madison, Wis.

the number born alive is reported in Table I. Parturition was uncomplicated in all cases and 90% of the young were born alive. A diet which contains 5% hydrogenated fat has been shown to be too deficient in essential fatty acids to allow normal lactation(11). However, in the present study the diet was supplemented with enough corn oil to successfully overcome any essential fatty acid deficiency.

A recent report by Alfin-Slater *et al.*(13) that *trans* fatty acids are harmless was based on the results of feeding *trans* fatty acids to rats for 46 generations. The present results indicate that only traces of *trans* acids would be passed on to the fetus and each generation of rats would therefore start to accumulate them only after birth.

Johnston *et al.*(14) found human tissue to contain 2.0-14% of *trans* fatty acids. It would be of interest to know whether these *trans* fatty acids originated exclusively from dietary fat or were transferred through the placental wall, from a mother to her baby. Further studies in this regard are in progress.

Summary. Less than 0.5% of *trans* fatty acids were found in fat extracted from young born to mother rats which contained between 23.5 and 26.8% *trans* fatty acids in their carcass fats. The amount of *trans* fatty acids in the carcass fats of the young was markedly increased when they were allowed to suckle the maternal milk for 9 days.

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Immunogenicity of Particles Isolated from *Mycobacterium tuberculosis.* (23602)

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Youmans, Millman, and Youmans(1) have reported that mice immunized with very small enzymatically active particles (20-200 mμ) isolated by ultracentrifugation from extracts of *Mycobacterium tuberculosis* var. *hominis,* strain H37Ra, were as resistant to tuberculous infection as were mice immunized with whole living cells of the same strain. Since this first report, work has continued on the chemical(2,3), physical, and biological characteristics of these particles, and attempts have been made to isolate similar immunogenic particles from the BCG-4 strain of *Mycobacterium tuberculosis* var. *bovis.*

It has been found, using an extraction procedure similar to the one previously outlined (1), that consistently immunogenically active preparations can be obtained from the H37Ra strain. Greater difficulty has been encountered, however, in obtaining consistently immunogenically active preparations from the BCG strain.

The present paper will be concerned with

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some of the factors involved in the preparation of immunogenically active particles from both the H37Ra and BCG strains of *Mycobacterium tuberculosis*, with the chemical nature of the particles and with the effect of various treatments on the immunogenicity of the particles.

Methods. Particles to be used for immunization were extracted from 2 strains of mycobacteria: the attenuated human type strain, H37Ra, and the attenuated BCG strain, number 4.† The H37Ra cells were grown as surface pellicles on 300 ml of modified Proskauer and Beck medium(4) contained in one liter flasks, and were harvested at the time of mature growth (21-35 days) by pouring the contents of the culture flasks into coarse sintered glass filters, and washing the collected cells with 0.01 M phosphate buffer (pH 7.2). Growths from approximately 60 culture flasks were needed to give sufficient cells for the 6 grinding bottles usually employed for each preparation. The BCG-4 cells were grown on Sauton's medium in a manner similar to that used in the preparation of BCG vaccine(5). The age of these cells was approximately 14 days. Both freshly harvested and frozen BCG cells were employed to determine whether active particles could be obtained also from frozen cells which then would permit "stockpiling" of cells.

It was found as the studies progressed that more active immunogenic particles were obtained when smaller relative amounts of powdered glass were used for the grinding of the cells. Therefore, into each 250 ml heavy pyrex glass centrifuge bottle was placed 25 g of washed powdered glass, 200 g of stainless steel balls of different sizes, 40 g of the washed harvested cells, and 50 ml of sucrose buffer (0.25 M dissolved in 0.01 M phosphate buffer, pH 7.0). The rubber stoppered bottles were rotated for approximately 18 hours at 4°C on a ball mill. The extracts so prepared were fractionated by procedure described previously in detail(1). The high speed (40,000 rpm) sediment which appeared was composed of particles which were used to immunize the mice. This sediment after being carefully scraped from the edge of the tubes with a glass rod and homogenized gently by hand with a Teflon homogenizer was diluted with 0.25 M sucrose buffer to a volume of 5 ml for each grinding flask used, since this concentration protected approximately 50% of the mice. Throughout the preparations, it was necessary to keep all materials cold (4°C), and to use chemically cleaned glassware since trace amounts of detergent appeared to inactivate the particles. The sediment, when prepared from H37Ra cells, appeared as a red pellet of about 1 cm in diameter in each centrifuge tube and consisted of 2 layers: a loose top layer and a darker gelatinous bottom layer which adhered tightly to the tube. Because of this red color it has been termed the "red fraction." In contrast, however, the sediment prepared from BCG cells, while also consisting of the 2 layers, appeared grey in color. The immunogenicity of the individual layers was measured, and all except the bottom gelatinous layer prepared from BCG cells were found to be active. Groups of 20 mice, "Strong A" strain, ranging in weight between 18 and 22 g, were vaccinated by injecting intraperitoneally 0.2 ml of each of the homogenized suspensions of particles within 2 to 3 hours after their preparation. Four weeks later the mice were challenged intravenously with 1 mg, wet weight, of a standardized ground suspension of the highly virulent human strain, H37Rv(6). The time of death, in days, of each mouse was recorded until the 30th day, and an autopsy done to confirm that death was due to pulmonary tuberculosis. At 30 days all living mice were sacrificed. Two methods were used to evaluate the immunogenic activity of the fractions, and the choice of method was determined by the rapidity with which the mice died. When the mice died before 30 days a normal distribution curve of the deaths is obtained and Litchfield's method of determining the median survival time (St_{50}) can be used(7). Ninety-five % confidence limits then can be calculated and the results compared statistically. However when the immunized mice tended to

† BCG-4 cells were kindly supplied by Dr. Sol R. Rosenthal, Tice Clinic, University of Illinois, Chicago.

TABLE I. Immunogenic Effect of Whole Cells and of "Red Fraction" from Extracts of H37Ra Cells against Tuberculous Infection.

	Red fraction				Whole cells	
Age of cells in days	mg N/ml†	No. organisms per 0.2 ml (× 1000)	No. of mice	% No. of mice S-30*	No. of mice	% No. of mice S-30*
35	1.84	4.4	20	45.0	20	50.0
27	1.31	3.2	18	88.0	20	75.0
25	1.32	3.0	20	55.0	19	68.4
20	2.82	2.0	18	33.3	20	75.0
30	1.56	4.4	20	50.0	20	70.0
32	2.23	2.0	19	26.3	18	38.8
32	1.43	.4	18	44.4		
32	2.66	1.4	20	55.0	18	11.7
22	2.11	2.6	9	55.5	19	73.6
28	1.58	1.4	19	57.8	20	85.0
27	1.31	4.2	18	50.0	19	63.1
23	1.89	6.0	19	52.6	17	82.3
26	1.74	6.8	17	47.0		
			16	56.2		
26	2.01	4.8	18	61.1	16	43.7
		Total	269		226	
		Mean		51.8		61.4

* No. of mice which survived 30 days.

† Nitrogen determinations made on unwashed particles so traces of supernatant may be present.

die over a prolonged period of time and a normal distribution of deaths was not obtained, neither the median survival time nor the mean survival time could be used as a measure of the response to infection. For this reason, the number of mice which survived 30 days was used as a measure of immunity to tuberculous infection since 95% or more of the control infected mice are dead by this day. By employing the chi square test, mice vaccinated with various fractions can be compared statistically with the controls, or with each other. The methods used for the vaccination of mice and for the evaluation of the results have been described in detail in separate publications(6,8). Nitrogen content was determined on each sample of the standard dilutions of the "red fraction" by the micro-Kjeldahl technic(9) and the mg of nitrogen per ml of each "red fraction" was calculated. Bacterial counts were made also on each "red fraction" by spreading 0.05 ml of the standard dilution over an area of 5 x 1 cm on a microscope slide. The number of acid fast bacteria in 1,000 fields was counted and from this figure the number of whole cells remaining in the preparation could be calculated.

Results. Table I lists the results of 14 consecutive experiments in which "Strong A" mice were immunized with the "red fractions" obtained from freshly harvested H37Ra cells, and the living H37Ra cells from which the particles were isolated. It is apparent that both the "red fraction" and the whole cells were highly immunogenic in that they protected mice against a large challenging dose of virulent tubercle bacilli, and, moreover, the results were remarkably consistent, especially in mice vaccinated with the "red fraction."

Also included in the Table is the age of the cells employed in each experiment, the nitrogen values of each of the "red fractions," and the number of whole cells in the 0.2 ml of the "red fraction" injected into each mouse. There was no direct correlation between the immunogenic activity of the particles with either the nitrogen value, or the age of the cells from which they were prepared. The number of microorganisms present in each sample was far too small to immunize alone, as it had been found previously(1) that between 1,610,000 and 17,381,000 organisms of the H37Ra strain were needed to produce a detectable immune response in this strain of mice.

The effect of dilution, heat, lyophilization,

TABLE II. Effect of Physical Factors on the Immunogenic Activity of the "Red Fractions" Obtained from Extracts of H37Ra Cells.

Treatment	No. of mice	No. of replicate exp.	D-30*	S-30‡	% S-30	Median survival times (St_{50}) (95% confidence limits)	
Red fraction	269	14	130	139	51.8		
1- 5 dilution	18	1	18	0	.0	19.5 (18.4-20.6)	S†
1- 10 "	146	8	124	22	15.0	16.0 (15.5-16.5)	S
1- 20 "	20	1	18	2	10.0	16.0 (15.2-16.9)	S
1-100 "	53	3	50	3	5.6	15.0 (14.3-15.8)	
Red fraction—heat (126°C for 15 min.)	70	4	66	4	5.7	16.5 (16.0-17.0)	S
1- 5 dilution	20	1	19	1	5.0	18.5 (17.5-19.6)	S
1- 10 "	73	4	71	2	2.7	16.0 (15.6-16.5)	S
1- 20 "	20	1	20	0	.0	16.0 (15.4-16.7)	S
1-100 "	37	2	37	0	.0	13.5 (12.9-14.2)	
Red fraction—lyophilized							
0.25 M sucrose buffer	66	4	43	23	34.8		
1- 5 dilution	19	1	16	3	15.8	17.0 (14.9-19.4)	S
1-10 "	57	3	54	3	5.2	16.5 (16.0-17.0)	S
1-20 "	19	1	18	1	5.3	16.5 (15.9-17.2)	S
0.88 M sucrose buffer	59	3	21	38	64.4		
Red fraction—sonic vibration, 2 hr, 9000 cycles	37	2	23	14	39.1		
Red fraction—Berkefeld filtered	40	2	37	3	9.2	15.5 (14.6-16.5)	
Control, non-immunized mice	251	14	243	8	3.1	14.5 (14.1-14.9)	

* No. of mice which died before 30 days. † S = A statistically significant difference between this median survival and that of the controls. ‡ No. of mice which survived 30 days.

sonic vibration and Berkefeld filtration on the immunogenic activity of the particles prepared from H37Ra cells is given in Table II. Dilution of the "red fraction" with 0.25 M sucrose buffer markedly reduced its immunogenic activity, although a 1-20 dilution produced in the mice a median survival time which was significantly longer than was found with the controls. The effect of other diluents is being investigated.

Since autoclaving H37Ra whole cells at 126°C for 15 minutes reduced their immunogenic activity about 50% (8,10) the particles were autoclaved in a similar manner. The immunogenic activity of the particles also was reduced markedly. The dilutions through 1-20 gave a slightly longer survival time in the mice than was found with the control mice, although the number of mice which survived 30 days was similar to the number of control mice which survived for the same period.

In each of 4 experiments when the particles were lyophilized in 0.25 M sucrose buffer significant immunogenic activity remained. However, lyophilization caused a decrease in activity from 51.8 to 34.8%, and this difference was found to be statistically significant at the 95% level when analyzed by the chi square test. Dilutions of the lyophilized material through 1-20 produced a slightly longer median survival time than was found with the controls. When the particles were lyophilized in 0.88 M sucrose buffer there was no decrease in activity.

Sonic vibration at 9,000 cycles for 2 hours decreased the immunogenic activity (S-30) of the particles from 51.8 to 39.1%. This was found also to be statistically insignificant. Filtration of the suspension of particles through a (German) Berkefeld filter, normal porosity, eliminated the immunogenic activity, possibly by retention of the particles. In addition, a number of experiments were conducted in which the "red fraction" was kept at 4°C for 24 and 48 hours, and one week before being used to vaccinate the mice. There was no loss of immunogenic activity following this treatment, nor following washing of

TABLE III. The Immunogenic Activity of Whole BCG Cells, Both Frozen and Freshly Harvested, and Particles Prepared from These Cells.

Vaccine	No. of mice	No. of exp.	D-30*	S-30†	% No. of mice, S-30
Particles prepared from fresh BCG cells	183	10	150	33	18.0
Original whole BCG cells	180	10	59	121	67.2
Particles prepared from frozen BCG cells	296	16	215	81	27.3
Original whole frozen BCG cells	280	15	96	184	65.7
None (controls)	380	20	363	17	4.4

* No. of mice which died before 30 days. † No. of mice which survived 30 days.

the particles 3 times by centrifugation with 0.25 M sucrose buffer at 40,000 rpm for 3 hours.

In contrast to the rather consistent results obtained with the "red fraction" prepared from H37Ra cells, particles prepared from freshly harvested BCG cells and frozen BCG cells varied widely in immunogenic activity. A summary of the results is given in Table III. These combined data indicate a very low degree of activity for the particles isolated from BCG cells although actually in individual experiments the percent of the mice which survived 30 days ranged from 0.0 to 42.1% when the particles were prepared from freshly harvested cells, and from 0.0 to 57.8% when the particles were prepared from the frozen cells.

In 2 experiments, particles prepared from frozen BCG cells and which permitted 49.5% of the mice to survive 30 days, were sonically vibrated at 9,000 cycles for 2 hours. These treated particles permitted only 20.0% of the mice to survive 30 days. This change was found to be statistically significant at the 99% level when analyzed by the chi square test. In addition, the immunogenic activity of the particles washed three times with 0.25 M sucrose buffer was reduced significantly. Thus, the immunogenic activity of the particles prepared from BCG cells appears to be affected more easily by these procedures than the activity of these particles prepared from H37Ra cells. It was noted also that the immunogenic activity of the particles prepared from BCG cells was reduced significantly when the particles were suspended in distilled water instead of sucrose buffer.

Nitrogen determinations and bacterial counts were made on each preparation and were similar to the values given for the "red fraction" prepared from H37Ra cells. With fractions from both H37Ra and BCG there was no direct relationship between nitrogen value and activity.

To determine the chemical composition of the immunizing "red fraction," the entire yield of particles from 14.0 g (dry weight) of *M. tuberculosis* var. *hominis*, strain H37Ra, was washed 5 times by centrifugation at 40,000 rpm for 3 hours in 0.25 M sucrose. The fraction was washed and resuspended in 0.25 M sucrose because it had been found that washing with distilled water liberated nitrogenous material and therefore reduced the yield. The washed fraction was lyophilized and weighed. The total yield, corrected for estimated sucrose, was 223.0 mg, dry weight. The fraction then was analysed according to the procedure of Schneider(11), and Table IV shows the results of this analysis. More than 70% of the fraction was composed of lipid. No desoxyribonucleic acid (DNA) was detected. Actually, over 90% of the DNA known to be present in whole mycobacterial cells could be found in the supernatant frac-

TABLE IV. Chemical Analysis of Immunogenic "Red Fraction" from *M. tuberculosis* var. *hominis* Strain H37Ra.

Chemical fractions	Wt (mg)	% of total
Total acid soluble P	.22	.1
" " " N	1.08	.48
Phospholipid A (alcohol soluble)	133.8	60.0
Phospholipid B (alcohol-ether 3-1 soluble)	28.0	12.6
Desoxyribonucleic acid	.0	.0
Pentosenucleic "	11.6	5.2
Protein	29.5	13.2
Total	203.98	91.58

tion, indicating almost complete solubilization of the nuclei. A quantitative determination of polysaccharides could not be made because of the presence of sucrose in the suspending medium. However, paper partition chromatography of the "red fraction" showed the presence of several carbohydrate components the nature of which is being studied at the present time.

Preliminary results of a similar chemical study of the immunizing fraction from BCG cells have produced essentially the same results.

Discussion. The frequent low degree of immunizing activity of the particles isolated from BCG cells when compared to those isolated from H37Ra strain is difficult to explain. Particularly since whole cells of BCG appear to be equal, if not better, immunizing agents than the H37Ra strain(8). The BCG fractions were prepared in the same manner as were fractions of H37Ra. However, there is a suggestion that the particles prepared from BCG cells may be more labile than the particles prepared from H37Ra cells.

The susceptibility of the immunogenic activity of the particles to various physical treatments was not unexpected in view of previous results which indicated that stabilizing agents were necessary to maintain maximum enzymatic activity of these same particles(2,3). This provides further evidence that these particles are "mitochondrial-like" since it has been shown that suspending fluids of high tonicity preserve the integrity of mammalian mitochondria. However, Marr and Cota-Robles(12) and Robrish and Marr(13) have shown that cell wall preparations from certain other microorganisms also might contain enzymes (hydrogenase and cytochrome). Unfortunately, the results of the chemical analysis of the isolated washed particles give little information concerning the relation of structure to immunogenic activity, but the relatively high lipid content of the immunogenic fraction might indicate that these particles may have consisted of cell wall fragments. However, Mudd *et al.*(14) have demonstrated by electron microscopy the presence in tubercle bacilli of intracellular "rosettes" which in size and susceptibility to electron bombardment resemble the immunogenic particles isolated in this laboratory. These findings appear to be evidence against the possibility that the particles found in the immunizing "red fractions" consist of cell wall fragments. Actually the enzymatic activity of the cell wall preparations mentioned above(12, 13) possibly may have been due to contamination by adsorption with components of disrupted intracellular particles. Since no DNA was found, it would appear that the immunizing fraction did not contain nuclei.

The marked difference in color noted between the immunogenic fractions obtained from the BCG strain and the H37Ra strain might be related to a difference in the amount of cytochrome. Since the chemical analyses performed showed no difference between the strains H37Ra and BCG, work is now in progress to determine whether immunogenic particles obtained from different strains of tubercle bacilli may differ in their content of cytochromes, and of purine and pyrimidine bases.

Summary. 1. By following a carefully standardized procedure highly immunogenic particles uniformly were obtained by ultracentrifugation from ground extracts of the H37Ra strain of *Mycobacterium tuberculosis* var. *hominis*. These particles, which ranged in size from 20-200 mμ, were just as effective as the living whole cells of this strain in protecting mice against a severe tuberculous infection. 2. The sediment comprised of the particles prepared from H37Ra cells was red in color and therefore has been termed the "red fraction." The immunogenic activity of this fraction was almost eliminated when the particles were diluted 5 times or more with 0.25 M sucrose buffer, autoclaved at 126°C for 15 minutes, or filtered through a Berkefeld filter. However, lyophilization in 0.25 M sucrose buffer significantly reduced their immunogenic activity while lyophilization in 0.88 M sucrose buffer did not. Sonic oscillation appeared to have no significant effect on the activity of the particles. When the cells were washed 3 times with sucrose buffer the immunogenic activity was not affected. 3. In contrast to these findings, even though the same preparation procedures were followed,

the fraction containing the particles when prepared from BCG cells was not always immunogenically active. The sediment containing the particles appeared grey in color, and the immunogenic activity was reduced by sonic vibration and by washing with sucrose buffer. 4. A chemical analysis of the particles from both H37Ra and BCG showed that they contain large amounts of lipid and no DNA. The significance of these findings is discussed.

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Vit. D and Citrate Metabolism. Inhibition of Vit. D Effect by Cortisol.* (23603)

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The search for metabolic effects of Vit. D which might throw light on its mechanism of action both in promoting bone salt deposition and in regulating serum calcium levels has led to studies of changes in concentrations of citrate in plasma, urine, and tissue associated with deficiency of Vit. D and its correction. Serum citrate levels are reduced in Vit. D deficient infants and are increased by Vit. D administration as is also urinary excretion of citrate(1). If large doses of Vit. D are given to rachitic children serum citrate levels are increased above normal range and hypercitricemia is seen as a manifestation of excessive Vit. D dosage both in patients(2) and experimental animals(3). Administration of Vit. D to Vit. D deficient rats increases the concentration of citrate in serum and in certain tissues such as kidney, bone and intestine although not liver(4). According to Carlsson and Hollunger(5) there is a slight initial decrease of citrate in serum of the Vit. D deficient rat during the first few hours post Vit. D and then a progressive increase which can be detected by 24 hours following Vit. D. The rise of serum citrate after Vit. D administration to some extent parallels the evidences of the antirachitic effect of Vit. D such as increase in serum calcium and phosphorus concentration and bone salt deposition. On the other hand feeding of calcium salts to Vit. D deficient rats given low calcium diets rapidly increases concentration of calcium but concentration of citrate in serum does not show a parallel rise(5). More recently DeLuca, Gran and Steenbock(6) have found that citrate is utilized more rapidly by rat kidney homogenates prepared from Vit. D deficient rats than from animals given Vit. D and they suggest that Vit. D retards conversion of citrate to α keto glutarate, thus leading to intracellular citrate accumulation. The asso-

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TABLE I. Effect of Cortisol on Concentrations of Phosphorus, Calcium and Citrate in Serum, and Calcium and Citrate in Bone of Rachitic Rats.

Group	Serum			Bone		
	Phosphorus	Calcium	Citrate	Calcium	Citrate	Citrate/Calcium
	mg/100 cc			mg/g		
Cortisol group	.8 ± .10 (10)	9.7 ± .17 (8)	1.7 ± .14 (12)	126 ± 3.16 (11)	3.53 ± .16 (11)	28.0
Rachitic controls	2.6 ± .18 (9)	10.1 ± .16 (11)	2.8 ± .18 (14)	109 ± 4.68 (10)	3.38 ± .20 (10)	30.8

Values are means and stand. error of the mean. Concentrations of citrate and calcium in bone are expressed as mg/g of dry fat-free bone. In final column the conc. of citrate in bone is also expressed as mg/g of calcium. Figures in parentheses indicate No. of animals on which the particular determination was made.

ciation of the effect of Vit. D on citrate levels in body fluids with its antirachitic properties has induced speculation that the citrate effect may be the primary effect of Vit. D and that complexing of calcium by citrate is of importance in the calcifying mechanism and in regulation of calcium concentrations of extracellular fluid.

Citrate levels in serum are reduced by cortisone and other steroids with similar biological properties such as cortisol and Δ_1 cortisol. The mechanism of this effect is unknown but it was thought possible to utilize this reaction to determine first whether the Vit. D effect on citrate was suppressed by adrenocortical steroids and second whether the antirachitic potency of Vit. D was influenced by interference with the citrate effect.

Procedure. Three-week-old male rats of the Sprague-Dawley strain were placed upon a synthetic high calcium, low phosphorus rachitogenic diet previously described(7). Following 2 to 3 weeks on this ration when skeletal changes are pronounced, one group of rats was continued without change, while another group was given this same diet to which cortisol was added in concentration of 30 mg/100 g diet. The steroid treated rats continued to eat amounts of diet comparable to the rachitic controls but failed to gain weight. After 10 days animals of both groups were given 100 units of Vit. D and continued on the respective diets while other animals were maintained on the two diets without Vit. D, as controls. Six to 8 days after Vit. D administration, the rats were anesthetized with sodium pentobarbital by intraperitoneal injection and blood obtained by puncture of the abdominal aorta. One tibia was taken for chemical analysis and the other for histological section. Determinations of calcium, phosphorus and citric acid were made on serum and on bone. Bone was dried to constant weight at 105° and defatted by extraction with ethyl ether. It was then dissolved in 8% trichloracetic acid and all analyses were made on trichloracetic acid filtrate. Calcium was determined in serum by a micro method(8) and in bone by the Clark and Collip method(9). Phosphorus was determined by the method of Fiske and Subbarow(10) and citrate by the method of Natelson, Pincus and Lugovoy(11). Tibia for histological section was fixed in 10% formalin, then embedded in celloidin and sections of the proximal third cut without decalcification. Sections were stained with silver nitrate followed by hematoxylin and eosin to reveal the pattern of calcification.

Results. Concentrations of calcium, phosphorus and citrate in serum and bones of Vit. D deficient rats on the rachitogenic diet with and without cortisol are shown in Table I. The low serum citrate levels characteristic of Vit. D deficient rats are further reduced by steroid feeding. Serum calcium concentrations are not altered by steroid feeding but serum phosphorus values are markedly reduced—so low in some animals as to be hardly measurable. Analyses of bone show an actual increase in calcium content of bone of the cortisol treated rats in comparison with control rachitic animals. This is of interest in light of the histological findings in bone discussed below. Citrate concentrations in bone of treated rats are somewhat higher than in controls when values are expressed in terms

TABLE II. Effect of Cortisol on Concentrations of Phosphorus, Calcium and Citrate in Serum, and Calcium and Citrate in Bone of Rachitic Rats Given Vit. D.

	Serum			Bone		
	Phosphorus	Calcium	Citrate	Calcium	Citrate	Citrate/Calcium
	mg/100 cc			mg/g		
Cortisol group	2.6	11.6	1.3	141	3.83	27.2
		10.6	1.5	105	2.46	28.7
		11.5	1.3	123	2.85	24.7
	3.5	12.1		143	3.53	25.0
	4.5	11.1	1.2	137	3.76	27.5
	4.5		1.3	132	3.54	26.8
Control Group 1	5.6 ± .15 (6)	11.5 ± .13 (7)	4.3 ± .13 (7)	130 ± 4.13 (7)	6.09 ± .35 (8)	46.8
Control Group 2	6.1 ± .82 (4)	11.6 ± .24 (4)	5.1 ± .31 (4)	177 ± 7.9 (4)	8.19 ± .44 (4)	46.2

Values in individual rats of cortisol fed, vit. D treated group are compared with mean values of control vit. D treated group (control group 1) and also with mean values of a group of rats which was given vit. D prophylactically in dosage of 100 units/wk while on the rachitogenic diet (control group 2). The data are expressed in the same units as in Table I.

of dried fat-free bone but are reduced below controls when expressed as mg/g of bone calcium. This latter concentration is probably the more significant since it indicates concentration of citrate in the crystals of bone salt. Table II compares similar determinations in Vit. D treated rachitic rats with and without cortisol feedings. For comparison findings in rats given Vit. D prophylactically are also shown (Control Group 2). The obvious finding is the marked reduction of serum citrate concentration in the cortisol group. The Vit. D treated controls show the expected increase of citrate values both in serum and bone. In cortisol fed animals serum citrate levels are actually lower than in D deficient rats despite Vit. D treatment. An increase in concentration of calcium and phosphorus in serum due to Vit. D occurs, however, despite suppression of the citrate effect although serum phosphorus values of cortisol fed rats remain somewhat lower than in controls. Concentrations of calcium in bone are the same in cortisol fed and control Vit. D treated rats. Concentrations of citrate in tibias of control animals are, however, much higher than in those of the cortisol treated group. Administration of Vit. D to control rats resulted in an increase of bone citrate parallel to the increase of serum citrate levels whereas cortisol administration suppressed this Vit. D effect both on serum citrate and bone citrate concentrations. Suppression of the citrate effect did not inhibit healing of the rickets, however, as indicated by histological changes in bone.

Typical sections of proximal tibias of rats of various groups are shown in Fig. 1-3. Tibia of cortisol fed, Vit. D deficient rat shows a retardation of cartilage growth which is in accord with suppression of weight gain. The overall architecture, however, is that of severe advanced rickets, *viz*: accumulation of proliferative cartilage, degenerative changes in this cartilage, and on the shaft side irregular in-

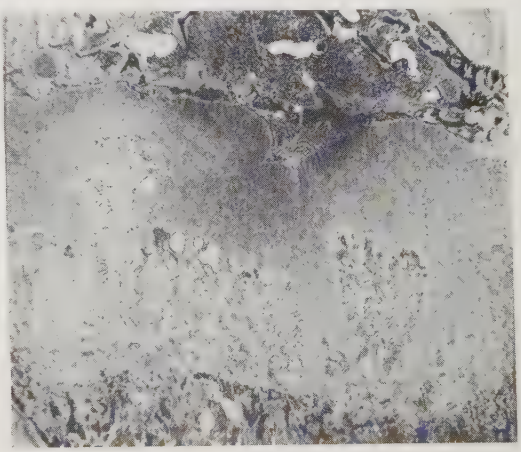

FIG. 1. Section of tibia of control rat fed rachitogenic diet. In this and subsequent Figures the epiphysis is at the top and the metaphyseal bone at bottom of photomicrograph. Magnifications are the same in all Figures (× 17). This section shows the extremely broad zone of uncalcified proliferative cartilage and accompanying degenerative changes.

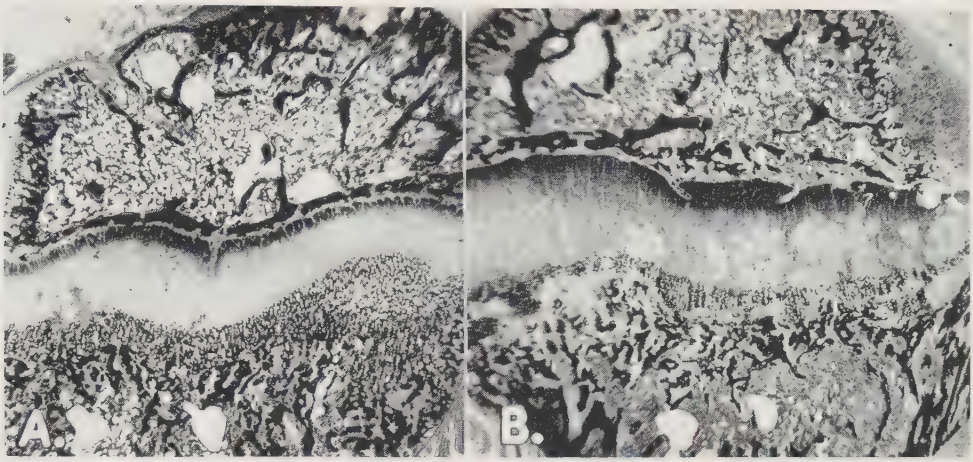

FIG. 2. Sections of tibias of rats given cortisol supplement without vit D. Inhibition of cartilage growth is shown by narrowing of proliferative cartilage. There is also a zone of calcification in the cartilage which borders on the shaft.

vasion of cartilage by vascular elements which sprout into it from the shaft at scattered points with tongues of cartilage in between. The trabeculae also bear witness to severity of the rickets by heavy encasements of osteoid. The most conspicuous difference from the severe rickets of control rats is that in tibia of cortisol fed rats the cartilage mass, which borders on the shaft, is heavily calcified. Calcification extends into the horizontal partitions of matrix separating cells from each other so that many individual cells are surrounded by calcium phosphate precipitate. It is of interest that cartilage cells in the calcified zone are intact with well preserved nuclei since in normal sequence the cartilage cell dies as the surrounding matrix becomes calcified under influence of invading capillary osteoblast complex. There is, further, little evidence of invasion by the capillary osteoblast complex into the calcified zone preliminary to its transformation into bone. In the shaft osteoid encasements of trabeculae are thick. Slight depositions of calcium phosphate may have occurred here and there in the osteoid. It is difficult to estimate osteoblastic activity

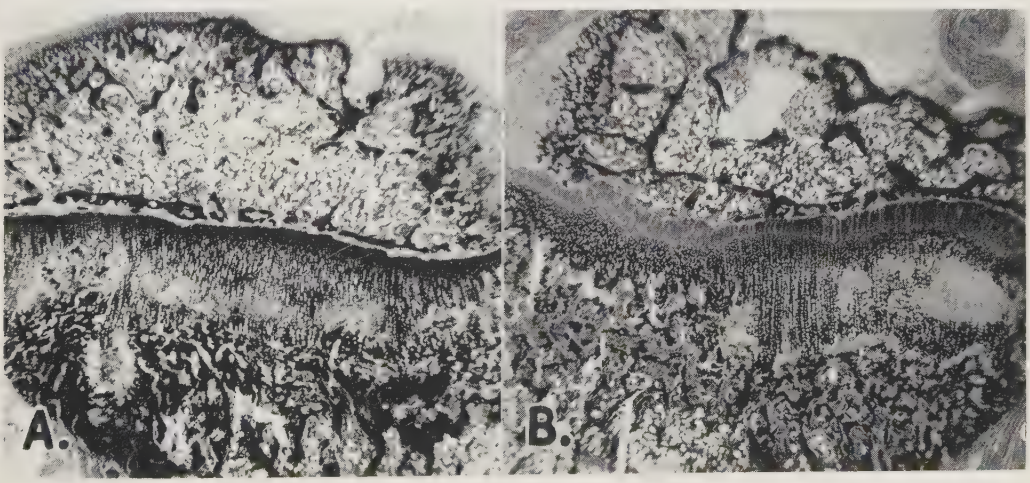

FIG. 3. Sections of tibias of rats given cortisol supplement and treated with vit D. Calcification of cartilage occurred in 2 zones, one just above the metaphyseal bone and another more distal at usual site of calcification in healing rickets. At some points the 2 zones have coalesced. These sections show both the growth inhibitory effects of cortisol and the antirachitic effect of vit D.

in these preparations but it appears that the osteoblastic coverings of the trabeculae are scantily developed and few osteoclasts are seen so that both formative and destructive aspects of cellular activity have been inhibited.

Tibias of cortisol fed rats treated with Vit. D show a characteristic zone of calcification high up in the proliferative cartilage, in the zone where healing normally occurs whether induced by Vit. D, citrate feeding or starvation. In all but one animal a fragmentary zone of calcification at the level seen in the cortisol fed Vit. D deficient rats can also be distinguished. The separate existence of this zone is obliterated in places where the zone of calcification resulting from Vit. D treatment has become broad enough to coalesce with cortisol induced calcification. In the one rat failing to show two separate zones of calcification the probable reason is that coalescence is complete. In the shaft the osteoid is calcifying as shown by diminished width of osteoid borders and deposits of streaks and sheets of precipitate in its substance. Capillary and cellular activity also appear to have been somewhat stimulated and there are signs of capillary invasion in the metaphysis, rows of osteoblasts are more prominent, and osteoclasts are also easily found. Some osteoblasts and osteoclasts are strikingly large. Administration of Vit. D to cortisol fed rachitic rats appears to have visibly increased cellular activity both of formative elements and destructive elements of bone.

Discussion. Feeding of cortisol in doses which inhibit growth reduces the concentration of citrate in serum and bones of Vit. D deficient rats and suppresses the enhancing effect of Vit. D on serum and bone citrate. Despite prevention of the citrate effect of Vit. D, cortisol does not block the antirachitic action of physiologic doses of Vit. D as measured both by rise of serum calcium and phosphorus concentrations and by calcification of rachitic cartilage. An antivitamin D action of cortisone has been suggested because of its effect in lowering the elevated serum calcium levels of patients with sarcoidosis and hypervitaminosis D(12). The present studies indicate a dissociation between antirachitic property of Vit. D and its effect upon citrate concentrations in body fluids and demonstrate that changes in serum and bone citrate levels are not a necessary concomitant of the healing of rickets. Citrate accumulation in bone may simply represent citrate uptake by bone crystals in contact with extracellular citrate and in cortisol Vit. D treated rats bone crystal deposition occurs in the presence of low extracellular citrate concentrations. The source of the extracellular citrate increment which usually follows Vit. D administration remains unanswered. Increased content of citrate in kidney and intestine of Vit. D treated rats(4) and decreased utilization of citrate by kidney homogenates of these rats(6) suggest that increased extracellular citrate reflects intracellular accumulation of citrate. It is clear, however, that increased citrate concentrations in serum and bone are not prerequisites for acceleration of the calcification mechanism by Vit. D.

The marked hypophosphatemia of the cortisol fed Vit. D deficient rat cannot be ascribed to an effect of cortisol upon renal excretion of phosphate since on low phosphate diet the urine is free of phosphate both in controls and cortisol fed animals. The depletion of extracellular phosphate must result from diminished intestinal absorption of phosphate or increased tissue uptake. It is unlikely that soft tissue uptake of phosphate is increased in view of inhibition of growth resulting from cortisol. There is evidence, however, that bone uptake of phosphorus is greater in cortisol treated rats than in controls. Calcification in rachitic cartilage in these rats, despite extremely low levels of serum phosphorus suggests that inhibition of cartilage growth by cortisol is associated with some change in cartilage matrix which results in local precipitation of bone salt despite depletion of extracellular phosphate. Cellular processes of bone destruction and resorption of bone salt may also be inhibited by cortisol. Tibias of steroid treated rats are heavier in proportion to body weight and have a higher concentration of calcium and phosphorus than those of control rats. If total phosphorus in a single tibia is adjusted to body weight, the average tibial phosphorus/100 g of body

weight is 9.3 mg in Vit. D deficient cortisol fed rat in comparison with 5.9 mg in comparable control rats. Following Vit. D administration this value is 12.9 mg in cortisol fed rats and 8.1 mg in controls. Suppression of body weight increment by cortisol is thus not accompanied by proportionate decrease of bone salt mass. Histological sections indicate that cartilage cell proliferation is inhibited by cortisol, and also that osteoblasts and osteoclasts are less prominent. The cellular activity of bone tissue seems to have been checked so that resorption as well as formation of bone is depressed. In bones of Vit. D treated rats not only has calcification of proliferative cartilage and osteoid been initiated but also the inhibitory effect of cortisol on bone cell activity appears to have been overcome.

Summary. Correlation of the effect of Vit. D in augmenting concentrations of citrate in plasma and bone with its antirachitic action has been studied. Rats were made rachitic by feeding a Vit. D deficient diet which was also low in available phosphorus. Addition of cortisol to the diet of such rats reduced concentration of citrate in serum and also blocked the effect of Vit. D in increasing serum and bone citrate levels. Antirachitic action of Vit. D as measured by rise of serum phosphorus concentrations and by histological evidences of calcification of rachitic cartilage and osteoid was not suppressed by cortisol. The antirachitic action of Vit. D and its effect upon citrate metabolism can, therefore, be separated. The tibias of Vit. D deficient cortisol fed rats show evidences of increased calcification in comparison with rachitic controls which might in part be due to inhibition of bone resorption as well as retardation of cartilage growth by cortisol. This increased calcification in cortisol fed rats is associated with extreme depletion of extracellular phosphate.

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Rapidly Sedimenting Properties of Specifically Precipitating Component of a Hashimoto's Disease Serum. (23604)

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Evidence has been presented that chronic thyroiditis in humans may be the result of an autoimmunization process in which an individual produces antibodies against tissue components of his own thyroid. Thus, Rose and Witebsky have shown that thyroiditis similar to that in humans can be produced in dogs and rabbits by injecting the animal with a thyroid extract, either of other thyroids of the same species or even a portion of the individual's own thyroid(1,2). Roitt, Doniach, Campbell and Hudson have reported, moreover, that the serum of patients with Hashimoto's disease contains materials capable of

precipitating with human thyroid extract(3). Witebsky, Rose, Terplan, Paine and Egan have shown that the serum of patients with chronic thyroiditis agglutinates tanned sheep red cells coated with human thyroid extract (4). Presumably, these effects are due to antibodies produced against the patient's own thyroid. That patients with Hashimoto's disease have a high gamma globulin has also been reported(5), and this fits in with the thesis of autoantibody production.

We have had occasion to confirm the observation that serum from a patient with chronic thyroiditis diagnosed as Hashimoto's disease gives a precipitin reaction with an extract of normal human thyroid. Further studies of this serum were made which involved quantitative precipitation reactions, Ouchterlony double diffusion of serum and thyroid extract on agar plates, paper electrophoresis and ultracentrifugation studies. Our most significant finding has been that the antibodies reside in the heavy globulin fraction rather than the light globulin.

Materials and Methods. The serum used was that obtained from one patient at thyroidectomy. Her thyroid was reported by the pathology laboratory to be a typical example of Hashimoto's disease. A second portion of serum was obtained six months postoperatively. The patient was of blood group A. Clinical laboratory studies revealed a radioactive iodine uptake of 9%, B.M.R. of 10, thymol turbidity of 17 units; clinical gamma globulin determination of 2.7 units (normal 1.1). Thyroid extract was prepared from normal gland obtained at autopsy by homogenizing one part of wet tissue in five parts of 0.9% saline. The homogenate was permitted to stand overnight at 5° and was then centrifuged. The supernate was cleared by filtration and the extract was used in most of these experiments. In a few experiments involving agar diffusion, more concentrated extracts were prepared using one part wet tissue to either equal or four parts of saline. Borate buffer used was of pH 8.0 and ionic strength 0.16 M. It was made by dissolving 10.3 g boric acid, 7.8 g sodium chloride, and 1.1 g sodium hydroxide in water to 1 liter volume. Protein concentrations of the solutions used were determined by micro-Kjeldahl analysis. The protein in the precipitates obtained in the precipitin reaction was determined as follows: Precipitates were washed once with 3 ml borate buffer and twice with 3 ml saline and then dissolved in 0.02 N NaOH. The solutions were transferred to the calibrated micro Beckman cells and diluted to 0.4 ml with additional sodium hydroxide. Readings were made in a Beckman DU spectrophotometer at 280 mμ(6). A conversion factor of 1 optical density unit = 0.775 mg of protein per ml based on the nitrogen content of human gamma globulin solutions times 6.25 as obtained by Kjeldahl analyses was applied. The conversion factor for thyroid extract was the same as for globulin in borate buffer and in 0.02 N NaOH. Ouchterlony double diffusion plates were prepared according to the modification described by Korngold(7). Four stainless steel penicillin assay cylinders were arranged at the corners of a square around the fifth central cylinder at a distance from the central cylinder of 2.5 cm. Merthiolate (Lilly) 1 part/10,000 was used in the agar as a bacteriostatic agent. The reservoirs were filled with 0.2 ml of serum or extract and incubated at 25°.

Results. Precipitin reaction. When portions of the Hashimoto's disease serum were added to successive dilutions of thyroid extract, a typical precipitin curve was observed. The amounts of precipitate formed at optimum were of the order of 3 mg/ml of serum. The amount of precipitate obtained passed through an optimum and when the supernates were tested with additional antibody or antigen, additional precipitates were observed in the classical antigen excess or antibody excess regions. Serum taken six months postoperatively gave no precipitate with thyroid extract. Neither did normal serum give precipitate with extract.

Ultracentrifuge studies. The sera obtained at thyroidectomy and six months later were analyzed ultracentrifugally in an analytical Spinco ultracentrifuge (Type E). Pictures were taken at 4 minute intervals and showed a large number of components with negative s-values (rapidly rising components) in the Hashimoto's disease serum, presumably lipo-

TABLE I. Protein Precipitated from Hashimoto's Disease Serum and Serum Fractions by Thyroid Extracts. 0.2 ml serum fraction and 0.2 ml of thyroid extract used.

Serum fraction	Serum protein used, μg	Thyroid extract added, μg protein: 34	69	137	274
		Protein precipitated, μg*			
Upper layer	3000	4	1	6	4
Lower "	3600	12	28	60	49
Pellet	1800	20	41	59	22
Hashimoto serum ⅓ conc.	5000	23	60	126	175

* Results are avg of duplicate tubes. Values are amt of precipitate above control values of 11, 12, 8, and 10 for serum fractions and saline as listed.

protein, even though the patient had not eaten for 12 hours. Serum taken six months post-operatively did not show these negative s-value components.

To determine sedimentation property of the precipitating component, three 1 ml portions of serum were centrifuged at 60,000 rpm for 53 min in a Spinco analytical ultracentrifuge with a preparatory rotor and partition cell until the heavy globulin peak had just crossed the partition. This insured that all heavy globulin components were in the lower compartment. The light globulin boundary had just become visible at the surface. The upper and lower compartment solutions were removed and the pellet taken up in a minimum amount of saline. The corresponding fractions of the three runs were pooled and made up to 3 ml. These three fractions, upper compartment, lower compartment, and pellet along with a dilution of the original serum (⅓ original concentration) were tested for precipitation with thyroid extract. Precipitin tests were set up in duplicate with 0.2 ml of two-fold dilutions of thyroid extract and an equal volume of serum or serum fraction. Dilutions of antigen were selected on the basis of a preliminary precipitin test. Tubes were incubated at 36° for 1 hr. and then at 5° for 2 days. Protein analyses were run on precipitates (see experimental). The results are given in Table I. It can be seen that the bulk of the precipitate was obtained with the pellet and with the bottom compartment contents. These two fractions contained the rapidly sedimenting components. The upper phase which showed only a trace of precipitin contained essentially the same concentration of globulin as the bottom phase, but did not contain any of the heavy globulin. Therefore, the bulk of the precipitin activity must have been associated with the heavy components rather than with the normal antibody of molecular weight 160,000 and s value of about 7.0 S.

Ultracentrifuge study of the pellet and lower compartment fraction revealed appreciable quantities of rapidly sedimenting globulin in both fractions.

Relative gamma globulin contents of the various solutions used in the precipitin reaction (Table I) were determined by simultaneous paper electrophoresis analysis of these solutions and comparing the areas of the gamma globulin peaks. The areas showed that the upper, lower and pellet solutions as used contained 42, 57, and 53%, respectively, of the amount of gamma globulin present in the original serum of ⅓ concentration as used. This emphasizes that the differences in amounts of precipitate obtained with these fractions cannot be due to difference in gamma globulin contents.

Paper electrophoresis studies were also made on the serum obtained 6 months post-operatively and on normal control sera. It can be seen from the results in Table II that the gamma globulin/albumin ratio of the top, bottom and pellet fractions increase in that order. Moreover, the gamma globulin/albumin ratio of the original Hashimoto's disease serum was greater than that of the 6-month post-operative serum and of several normal human sera run simultaneously. This is in line with observations made by others(8).

Ouchterlony double diffusion technic. A line of precipitate between reservoirs containing serum and undiluted extract appeared after 12 days when 1.5% agar was used. The line formed was closer to the antibody than the antigen reservoir, indicating a slower diffusion of antibody than antigen. The slower diffusion was not due to a low antibody concentration (see precipitin reaction). This is further indication of the high molecular weight of the antibody. Reservoirs with con-

centrations of ½, ¼ and ⅛ of the original thyroid extract had been set up on the same plate and all these dilutions showed lines by the 17th day. In order to speed up diffusion rate, the agar concentration was dropped to 1.2%. Lines formed in 6 days and were essentially equidistant from the reservoirs. The rate of formation of lines in this system was much slower than in the case of the ovalbumin-rabbit anti-ovalbumin system where lines formed in three days on similar plates. This again is evidence for the slow diffusion of the precipitating component of human serum.

Single lines were the rule, although in a few cases double lines appeared. These may have been due to artifact. More concentrated thyroid extracts (1 part tissue to 4 parts saline or to 2 parts saline) were also tested. Both reacted similarly with Hashimoto's serum and gave only one precipitation line.

Ouchterlony test of the sera obtained 6 months post-operatively gave a faint line in one run but no lines were observed in other attempts. Precipitin reactions using whole serum were negative.

To test the specificity of the reaction against other human sera, plates were prepared with undiluted thyroid extract in the center reservoir and a Hashimoto's disease serum and samples of normal sera of blood groups O, A, B and AB in the other reservoirs. Precipitation did not occur with any of the sera tested except that from the Hashimoto's disease patient.

TABLE II. Relative γ-Globulin/Albumin Ratios* for Hashimoto's Disease Serum as Determined by Paper Electrophoresis.

	γ-globulin / Albumin
Upper layer	.88
Lower "	1.18
Pellet	2.57
Original whole serum	1.09
Serum after six mo	.70
Normal serum No. 1	.78
" " " 2	.60
" " " 3	.36

* These ratios were calculated from relative areas of the peaks of the densitometer tracing of dyed paper electrophoresis strip and are not absolute values due to trailing errors inherent in paper electrophoresis. Correction has been made for the more intense staining of albumin by the dye on an equal weight basis. Human albumin is stained to an intensity of 1.4 times that of human globulin. A Spinco paper electrophoresis apparatus was used. Veronal buffer pH 8.6; $\Gamma/2 = 0.075$; 75 V; 16 hr at room temp.; stained with bromophenol blue and scanned with an Analytrol photoelectric scanner.

Discussion. In normal human serum ultracentrifugation schlieren patterns(9), there are generally 2 well resolved globulin peaks. The larger peak corresponds to gamma globulin with an s value of about 7.0 S and the smaller peak to gamma globulin of higher molecular weight with an s value of approximately 19-20 S. In Hashimoto's disease serum, we have found that the serum components, which are precipitated by thyroid extract and which are considered to be auto-antibodies, are associated with the heavy globulin fraction rather than the light globulin fraction and these are therefore in the class of the complete Rh antibodies(10) and some of the antibodies of the Wasserman type(11).

That they are of the heavy fraction is evidenced by two types of experiments. Ultracentrifuge studies showed that the precipitating protein sedimented with the components of value 20 S. Ouchterlony double diffusion experiments also indicated that the antibody was of a high molecular weight and slow in diffusing since the lines in this system were slow in forming.

Summary. Serum was obtained from a patient with Hashimoto's disease at thyroidectomy and six months post-operatively. The first serum was found to form a precipitate on the addition of thyroid extract as has been described by others. On ultracentrifugation, it was found that the precipitating components were in the heavy globulin fraction (s = 20 S) rather than in the light globulin fraction (s = 7 S). Double diffusion experiments by the Ouchterlony method also indicated that the antibody was of high molecular weight. The antibody seemed to have disappeared from the serum within six months after surgery.

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Stimulation of ACTH-Release in Humans by Non-Pressor Fraction from Commercial Extracts of Posterior Pituitary.* (23605)

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Isolation of a purified material of hypothalamic origin which stimulates release of ACTH *in vitro* was recently reported from this laboratory(1). Similar activity was found in the corresponding fraction when commercial extracts of posterior lobe of the pituitary were subjected to the same fractionation procedures. Evidence has been obtained that the active material is a small peptide different from vasopressin and oxytocin (1). The physiological significance of these findings as well as the composition and structure of the active factor are still to be elucidated. Even at this stage of fragmentary knowledge, it was of interest to investigate whether or not the hypophysiotropic fraction would stimulate the release of endogenous ACTH in humans. Stimulation of ACTH-release in the human has never been reported in the absence of stressing conditions such as fever, pain, discomfort, surgical procedures, electroshock, insulin shock, forced muscular exercise or emotional upset. The active hypophysiotropic material appeared devoid of any side effects on the basis of pharmacological studies(1) and produced increases of the plasma 17 hydroxycorticoids when administered to dogs(2,†). It was therefore decided to study its possible ACTH-releasing activity in humans without regard for the explanatory mechanism, *i.e.*, directly hypophyseal (specific) or trans-hypothalamic (non-specific), of any positive result as long as it would have been obtained in the absence of stress or general discomfort.

Material and methods. Two types of clinical investigations were carried out in a total of 36 human subjects. a. 5 to 10 mg of a material obtained by chromatographic fractionation of oxycellulose-washed Protopituitrin‡ and designated as fraction D(1), were administered by I.V. perfusion in 150 to 200 ml of saline over 4 hours to 14 hospitalized children (age 4 to 16 years) convalescent from various conditions. Determinations of the plasma "free" 17OH-corticoids (17-OHC) were done by a modification of the method of Nelson and Samuels(3) at times 0, 2, 4, 6 hours as a test of stimulation of ACTH-release. In some of these studies measurements of the urinary excretion of 17OHC by the method of Glenn and Nelson(4) were

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† Unpublished observations.

‡ Protopituitrin (Parke, Davis & Co.) is a crude acetic acid extract of post. pituitary (fraction C in Waring & Landgrebe's outline of Kamm fractionation procedure(7)). It is the commercial starting material for Pitressin® and Pitocin®.

TABLE I. Absolute Values and Variations of Concentration of Free Plasma 17OH-Corticoids in Subjects Receiving a 4-Hour Perfusion of Fraction D or an Identical Perfusion with Saline or Inactivated Material as Control.

	Time	0	2 hr	4 hr	6 hr
Fraction D	17OHC, μg %	12.7 ± 1.5	13.7 ± 2.0	27.1 ± 5.5	21.3 ± 12.9
	17OHC variations		+.1 ± 3.4	+14.4 ± 4.5	+9.3 ± 13.0
	No. of subjects	14	7	14	7
Saline controls or inactivated material	17OHC, μg %	18.2 ± 2.6	11.1 ± 4.1	10.5 ± 2.1	
	17OHC variations		−7.0 ± 3.8	−7.7 ± 3.5	
	No. of subjects	8	8	8	

carried out on aliquots of 24-hour urine specimens. Control studies were run on 8 patients with similar perfusions of saline or with inactivated fraction D (by alkaline hydrolysis). All perfusions were carried out between 7-8 a.m. to 11-12 a.m. The hypophysiotropic activity of the various batches of fraction D utilized in these studies was assessed *in vitro* (1) prior to administration. In all the 4-hour perfusion studies, the blood pressure was measured every 15 minutes by indirect sphygmomanometry for the duration of the infusion. Pulse, respiration, body temperature were checked and recorded as well as any subjective sign suspected or reported by the subjects. b. 25 to 45 μg of electrophoretically purified fraction DΔ(1) in 1 or 2 ml saline were administered in one single rapid I.V. injection to 5 adult healthy male subjects. Plasma free 17OHC concentrations were measured at time 0, 15, 30, 60 minutes. Simultaneous control studies were run with inactivated fraction DΔ. All materials were assayed *in vitro*(1) for stimulation of ACTH-release. By chromatography, electrophoresis and *in vitro* assays(1), fractions D and DΔ appear to have no ACTH contamination or activity. *In vivo,* fraction D (up to 1.5 mg) shows no ACTH activity by Sayers test in rats of 150-180 g or when injected (1 mg) in hypophysectomized dogs with measurement of adrenal vein 17OHC secretion(2). After adequate priming of the adrenal cortex with ACTH showing that the adrenals were responsive to stimulation, 20 mg of fraction D dissolved in 16% Armour USP gelatin solvent were given (5 mg, q-12 hr, 4x) to a patient with panhypopituitarism: urinary and plasma 17OHC levels fell abruptly during administration of this large dose of fraction D. Thus it can be reasonably ascertained that fraction D and DΔ have no ACTH activity or contamination at doses comparable or larger than those utilized here.

Results. A statistically significant elevation of the plasma 17OHC was obtained after 4 hours of perfusion with fraction D (Table I). Large variations in responses were observed. In spite of these variations even the *absolute* values of the plasma 17OHC at 4 hours are statistically different from those at time zero ($p \leqq 0.01$). It is possible that the 6 hour reading may indicate a residual stimulation of the pituitary-adrenal system if correlated with the corresponding values in the diurnal decline. A marked increase in the excretion of urinary 17OHC was observed in the 24-hour period starting with the administration of fraction D in 3 cases studied.

The rapid I.V. injection of fraction DΔ resulted in an increase of the plasma 17OHC after 15 minutes, with a gradual decline over 60 minutes. Due to the small sampling of the group receiving active material, this elevation is at the limit of statistical significance when compared against 17OHC concentration at time 0 in the same group. The observed increase is however statistically significant ($0.02<p<0.05$) when compared with the corresponding 15 minute values of the control group.

No objective changes were observed in the blood pressure, pulse, pulse pressure, cornea or skin color, skin moisture, respiratory rate or body temperature with any of the preparations administered. None of the subjects reported any feeling of general discomfort, nausea, intestinal cramp, or headache. The only remark sometimes made by the subjects

TABLE II. Absolute Values and Variations of Concentration of Free Plasma 17OH-Corticoids in Subjects Receiving One Intravenous Injection of 25-45 μg of Fraction D△ or an Identical Amount of Inactivated Material as Control.

	Time	0	15 min.	30 min.	60 min.
Fraction D△	17OHC, μg %	10.6 ± 2.7	16.8 ± 3.3	14.1 ± 6.0	12.0 ± 3.2
	17OHC variations		+6.2 ± 2.7	+4.5 ± 3.4	+1.4 ± 2.4
	No. of subjects	5	5	4	5
Controls, inactivated material	17OHC, μg %	11.9 ± 2.4	9.0 ± 1.5	8.9 ± 1.6	7.7 ± 1.7
	17OHC variations		−2.9 ± 1.1	−3.0 ± 1.5	−4.2 ± 1.0
	No. of subjects	9	9	9	9

receiving the acute injection of DΔ (in either active or inactivated form) was of a very mild sensation of warmth in the face lasting for a few seconds. Subsequently it was observed in the dog under Nembutal anesthesia with direct recording of the blood pressure through the carotid artery that rapid I.V. injections of 5 to 10 times the amount of DΔ given to the patients would produce a transient fall in carotid pressure. At the doses of DΔ given to the humans the effect was absent or non-significant. This transient vasodilatory effect was observed in the dog with active or inactivated DΔ and would appear to be due to an otherwise inactive contaminant.

Discussion. The limited number of experiments carried out with fraction DΔ was due to the conditions of its availability. So far the hypophysiotropic material is still made by paper chromatography and subsequent paper electrophoresis(1). The yields are extremely low and the reported lability of the electrophoretically purified fraction (as followed by the *in vitro* assay) has not been explained nor adequately controlled. Other methods of preparation of the highly purified fraction are being investigated. In this limited series of investigations a complete correlation of *in vitro* and *in vivo* results was obtained with both types of materials utilized. In contradistinction to the reports of McDonald *et al.*(5,6) these results show that stimulation of endogenous release of ACTH in humans as evidenced by a test based on the measurement of plasma corticoid concentrations can be obtained in the absence of painful side effects with a material extracted from Protopituitrin and devoid of pressor activity at the doses utilized here.

Summary. A non-pressor material, extracted from Protopituitrin, known to stimulate release of ACTH *in vitro* was shown to stimulate release of ACTH in human subjects as evidenced by measurements of plasma 17-hydroxycorticoids. This increased secretion of ACTH was obtained in absence of significant side effects.

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Blood Proteins and Their Distribution in Rats with Parabiosis Intoxication. Determined by Paper Electrophoresis.* (23606)

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Parabiosis intoxication is a condition which develops in animals united surgically. In rats the haematologic manifestations are severe anemia in one of the partners and a marked polycythemia in the other(1-5). Recent evidence from this laboratory indicates that one of the partners develops a state of vascular collapse, and as a result, gains blood at the expense of the other(6,7). It is suggested that the former attempts to reduce blood volume by storing red cells in the available reservoirs and diuresing water; the latter endeavors to increase blood volume by hemodilution with extravascular fluids. If this is true then polycythemic partners should show hyperproteinemia and anemic partners hypoproteinemia. Although anemic partners often show ascites, hydrothorax and subcutaneous edema, which might be construed as indicative of hypoproteinemia, direct data are not available.

It was the purpose of this study to determine the level of blood protein and the distribution of the various serum protein components in rats with parabiotic intoxication, in the belief that an understanding of the mechanisms underlying this syndrome might suggest an explanation for the high incidence of hypertensive cardiovascular disease(8-10), arthritis(11), and neoplasia(12,13) encountered in parabiotic rats.

Materials and methods. The rats used were from our colony, the forebears of which were of the Holtzman strain. The animals were maintained throughout the experiment in air-conditioned quarters and received tap water and Purina Laboratory Chow *ad lib.* The technic of parabiosis was as previously described(8), and was carried out on animals paired for sex and body weight (60-90 g) before union. The series consisted of 23 pairs, 20 female-female and 3 male-male pairs of comparable weight; and 8 single male and female controls. Hematocrit, hemoglobin and red-cell counts were determined by routine methods and when diagnosis of parabiosis intoxication was confirmed by these findings, the pairs were anesthetized with ether. Blood was drawn from the abdominal aorta of each of the partners and controls for analysis of the proteins. After withdrawing blood, the partners were killed with ether, carefully separated along the suture line and weighed. Controls were also weighed after withdrawal

TABLE I. Characteristics of the Blood in Single Rats Contrasted with Those of Rats with Parabiosis Intoxication.

Data	Single controls	Parabiotic pairs: Anemic twins	Parabiotic pairs: Polycythemic twins
Body wt, g—Initial	75 ± 2 *	85 ± 3	85 ± 2
Final	103 ± 2	85 ± 8	115 ± 7
Hematocrit, %	45 ± 1.4	20 ± 1.4	66 ± 1.9
Hemoglobin, g/100 cc	13 ± .3	6.5 ± .5	20.1 ± .5
Erythrocytes, millions/mm³	6.72 ± .23	3.28 ± .25	10.67 ± .43
Plasma protein, g/100 cc	6.10 ± .21	4.35 ± .70	7.96 ± .24
Serum proteins, % albumin	38.0 ± 2.1	27.3 ± 1.4	26.6 ± 1.7
α_1 globulin	13.0 ± 1.3	16.9 ± 1.1	16.6 ± .9
α_2 "	7.4 ± .6	16.5 ± 1.0	17.2 ± 1.6
β "	31.1 ± 3.0	31.0 ± 1.8	30.5 ± 1.9
γ "	10.6 ± 1.3	8.3 ± 1.0	10.3 ± .9

* ± S.E. of the mean.

* Aided by grant from Am. Cancer Soc.

Single Control, Normal

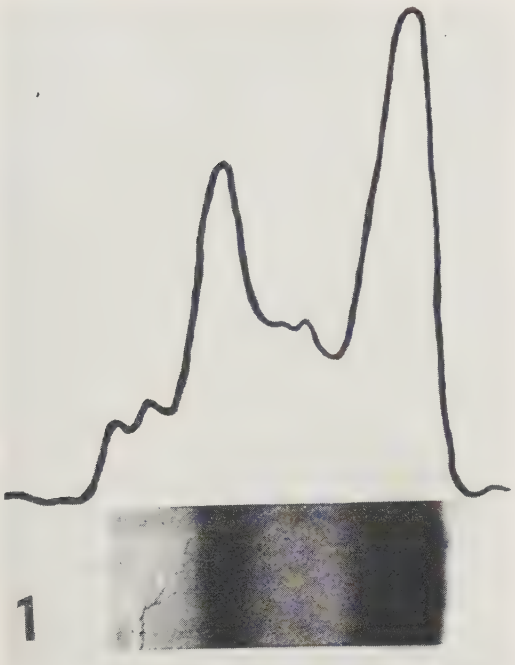

FIG. 1. Electrophoretic separation of serum proteins from a control rat. Plasma proteins 6.25 g/100 ml.

of blood. Plasma proteins were estimated by the method suggested by Lowry and Hunter (14), using plasma samples collected from a heparinized capillary tube after centrifugation. Blood samples were centrifuged and the plasma removed, allowed to clot, and 0.01 ml of undiluted serum was removed, subjected to paper electrophoresis and the protein components estimated by means of an Analytrol

Rat 1368—Left, Anemic

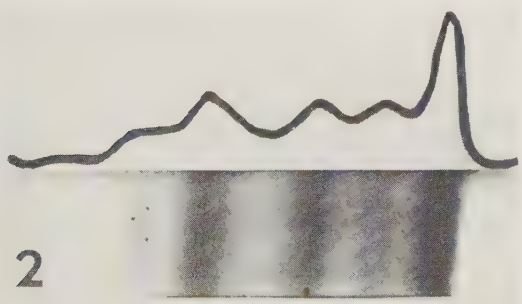

FIG. 2. Electrophoretic pattern of serum proteins from the anemic partner of a pair, #1368, with parabiosis intoxication. Plasma proteins, 1.75 g/100 ml.

recording scanner and integrator.

Results. The results are given in Table I. It will be noted that the hematocrit, hemoglobin and red cell counts of the control rats were within the limits established as normal for the species, and that a severe state of anemia and polycythemia respectively existed in the partners with intoxication. Plasma protein concentration was found to average 6.10 g/100 cc for the controls, in contrast with 4.85 and 7.96 g/100 cc respectively for anemic and polycythemic animals. Serum electrophoretic patterns of anemic and polycythemic animals as compared to controls, both showed a decline in the percentage composition of albumin, and a concomitant increase in the percentage of α_2 globulin.

Rat 1368—Right, Polycythemic

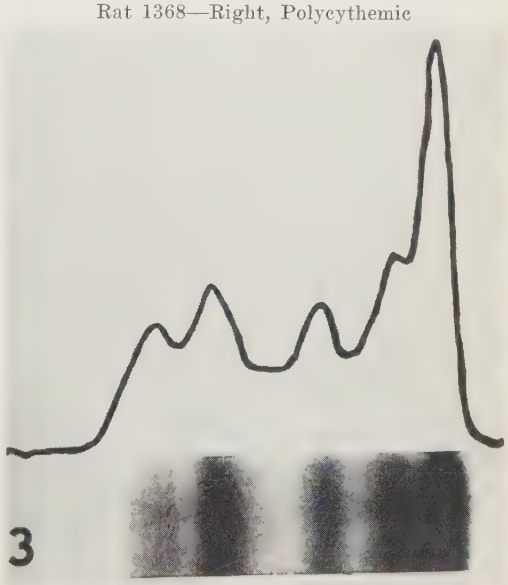

FIG. 3. Electrophoretic pattern of serum proteins from the polycythemic partner of pair #1368. Plasma proteins, 9.25 g/100 ml.

Discussion. The control values of both total plasma protein(15) and its percentage composition(16,17) were in general conformity with the figures reported for the rat. β globulin accounted for a greater percentage of the total than others have reported for the species but the significance of this is obscure in view of the known variability of serum protein electrophoretic patterns with age and strain(16,17). The animals utilized in this

study were very young, and such are known to have a proportionately greater serum β globulin level than older animals(17).

It is evident that the plasma protein concentration is augmented by about 30% in anemic animals and diminished by a comparable amount in their polycythemic partners. These findings are confirmatory of the inferences drawn as to their probable distribution from the physiologic derangements known to characterize animals with parabiosis intoxication(6). Furthermore, the fact that the changes in protein concentration are of comparable magnitude but opposite in direction from the normal values, is much easier to reconcile with the thesis that the fundamental disturbance is haemodynamic in nature, than with any of the proposed alternatives.

Despite the apparent differences in blood protein concentration in the partners, the percentage which each of the constituent proteins contributed towards the total was the same in anemic animals as in polycythemic. However, compared to normal values both partners demonstrated a decline in the percentage of albumin and a concomitant increase in that of the α_2-globulin. Such a pattern in man is said to be characteristic of conditions in which there occurs extensive and fairly severe tissue inflammation and destruction(18), which may well be the case in parabiosis intoxication. Statistical analysis revealed that the other serum protein partitions were normal in both polycythemic and anemic partners.

Normal parabiotic rats are said to show a diminished percentage of serum albumin and an augmentation of β globulins as compared with single controls, although total plasma protein concentration is unaffected(19). These data indicate that when intoxication supervenes there is an apparent change in total protein concentration favoring the polycythemic animal at the expense of its partner, and that in both the percentage of serum α_2-globulin is increased, and that of the albumin decreased.

Summary. Rats which have developed parabiosis intoxication show, in addition to the other hematologic aberrations which characterize the condition, changes in the blood proteins. Anemic partners have a low plasma protein level and polycythemic animals an elevated plasma protein titre. The partitions which albumin and the respective globulins contribute toward the total serum proteins average the same in both anemic and polycythemic twins, although as compared to control levels there is a diminished serum albumin and an elevated α_2-globulin component in each.

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Sodium and Potassium Contents of Frog Muscle after Extraction in 50% Glycerol.* (23607)

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It is well known that extraction of muscle in 50% glycerol leaves the contractile mechanism of the muscle intact but destroys the electrical excitability(1). The myosin itself, however, will still contract when exposed to ATP. It was of interest to discover to what extent this treatment also destroyed the ability of the muscle to concentrate potassium.

Method. Experiments were carried out with the assistance of Mrs. Sidsel Aksnes of Oslo, Norway. For this purpose a variety of small muscles were dissected from frogs (*Rana pipiens*). All the different muscles appeared to give the same results and they were used interchangeably. At first each muscle was tied to a glass frame to keep it at constant length during the glycerol extraction but this proved to be an unnecessary refinement. The duration of the extraction period was varied from 12 hours to several days without causing any change in the results. The muscles were weighed on dissection, after extraction and after subsequent incubation in the desired solutions. Changes in weight did not alter the significance of the results which are presented in terms of final weights. For analysis the muscles were either dry-ashed in platinum crucibles at 500°C or wet-ashed in nitric acid. The resulting solutions after suitable dilution were analyzed for Na and K on a Coleman flame photometer.

Results. The results, some of which are summarized in Table I, showed to our surprise that these muscles not only failed to concentrate potassium but they actually contained more Na than K even when the concentrations of both of these ions in the solution were practically zero. The ratio of Na/K varied from 1.32 to 3.9. Since this result might not represent a true equilibrium and might depend, therefore, upon the rates of extraction, it seemed worthwhile to re-equilibrate these glycerol extracted muscles in solutions containing equal concentrations of Na and K and to determine again the amounts retained. This has the added advantage that the glycerol is removed from the muscle in the process of equilibration so that the result cannot be influenced in any way by its presence. These results are shown in Table II. In all cases it is found that more Na than K is retained in the muscles. The smaller the concentrations of Na and K in the solution the greater, in general, was the Na/K ratio in the muscle. The muscles lost 20-35% of their initial weight during the process of extraction and subsequent equilibration in the KCl-NaCl solution. The total concentration inside the muscle is higher than that in the solution except in the higher concentrations, where the reverse is true. While the detailed and quantitative interpretation of these fig-

TABLE I. Sodium and Potassium Contents of Muscles Extracted in 50% Glycerol.

Time, days	No. of muscles	K, meq/kg	Na, meq/kg	Na/K
1	3	4.32	6.57	1.52
2	1	4.63	10.04	2.18
4	2	2.90	3.82	1.32
1	2	3.15	12.3	3.90

Calculations based on final weights after equilibration.

TABLE II. Average Na and K Contents of 50% Glycerol-Extracted Muscles when Equilibrated with NaCl + KCl Solutions of Equal Concentration.

Solution, conc. of KCl and of NaCl, meq/l	No. of analyses	Time, hr	K, meq/kg	Na, meq/kg	Na/K
55	8	.16-5.0	50.5	57.3	1.14
33	5	1-5	34.0	54.6	1.60
11	5	1-5	23.5	48.2	2.05
5.5	7	.25-2.0	16.5	26.2	1.59
2.75	3	.25-1	8.18	23.6	2.89

All calculations based on final weights after equilibration.

* This work was supported in part by a grant from Natl. Inst., Research Grants Division.

TABLE III. Na/K Ratios in Glycerol-Extracted Muscles under Different Conditions.

Condition	No. of muscles	Conc. in solution, meq/l Na_o	Conc. in solution, meq/l K_o	Time, hr	Ratio in muscle $\frac{Na_i/Na_o}{K_i/K_o}$ (S.E.)
10% muscle homogenate	7	15.3	18.2	.01–.18	1.49 ± .14
20 mg % ATP	4	11	11	.1 –.25	1.28 ± .07
pH 7	2	12	11	2	1.25
pH 6	2	12	11	2	1.50
pH 7	2	6	5.5	2	1.50
pH 6	2	6	5.5	2	1.59
2 mM $CaCl_2$	2	11	11	1 – 2.5	1.62
3 mM $MgCl_2$	2	11	11	1 – 2.5	1.18
Control	4	11	11	2	1.52

ures must await further experiments, it is abundantly clear now that under the conditions of these experiments at least there is no tendency for the myosin to prefer K to Na as a cation. To support the theory of preferential combination with K it will be necessary to show how the extraction with glycerol has altered the binding sites on the protein so that Na is preferred to K.

Some additional data are shown in Table III. It was thought that possibly the inability to concentrate K after glycerol extraction depended upon the loss of some normal muscle constituent. In an effort to replace this loss the muscles after glycerol extraction were incubated in a 10% muscle homogenate which had been cleared by centrifugation and to which equal amounts of KCl and NaCl had been added. After varying periods up to 18 hours the muscles were analyzed as well as the solutions. On the average the ratio of (Na_i/Na_o) (K_i/K_o) was found to be 1.44 ± 0.11. Evidently the ability to concentrate K was not appreciably restored by this treatment. Similarly no appreciable effect was obtained by a brief equilibration in a KCl-NaCl solution to which 20 mg % ATP had been added. Here the Na/K ratio in the muscle was 1.23 ± 0.08. Longer periods of equilibration might have proved more effective but were not tried because of the ATP-ase activity of the muscle.

In the last experiments of Table III it was found that the Na/K ratio was greater at pH 6 than at pH 7 and greater at lower concentrations of NaCl-KCl. Phosphate buffers were used for the pH control. None of these variations seemed to destroy the preference of the myosin for Na. $CaCl_2$ and $MgCl_2$ were also ineffective.

In an earlier preliminary study from this Laboratory an abstract was published(2) reporting a preferential combination of myosin with potassium. Later the same year in endeavoring to repeat this finding 3 experiments were tried in which a well washed preparation of myosin was equilibrated with a solution containing .095M KCl and .096M NaCl. After equilibration the protein was centrifuged out and the supernatant was analyzed. The ratio of Na/K in the protein was found to be 1.09. The result is not conclusive since a small excess of Na over K might have been present in the original protein in spite of careful washing although a few analyses of the final protein residue revealed nearly the calculated amounts. Some dialysis experiments were also tried in which the myosin was contained in and was dialyzed against the same KCl-NaCl solution used above. Analyses of the inside and outside solutions after several days revealed an inside/outside concentration ratio of 1.0 for K and 1.03 for sodium. Here again there was perhaps a slight tendency to concentrate sodium rather than potassium.

Some similar experiments were tried (in 1942) with a preparation of a structural protein, renosin, extracted from kidney as described by Szent-Györgyi(3). After careful washing of this protein in H_2O to remove electrolytes it was added to 4 vols. of a solution containing .095M KCl and 0.096M NaCl and allowed to equilibrate for 3 hours. Some of the protein dissolved but the remainder was

TABLE IV. Kidney Protein (Renosin) Suspended in a Solution of .095 M KCl and 0.096 M NaCl Showing Preferential Absorption of Na Relative to K.

Calculated in original protein		In original supernatant		In final protein		In final supernatant		Na_p/Na_s
K	Na	K	Na	K	Na	K	Na	K_p/K_s
2	8	760	768	67	88	695	688	1.22
34	43	760	768	40	64	754	747	1.62
(−10)	30	380	384	38	56	332	358	1.38
10	53	380	384	42	64	348	373	1.43
Mean Na/K 3.8		1.02		1.46		1.01		1.41

Figures for Na and K represent amounts in meq, not concentrations.

then centrifuged out and analyses were made on both the supernatant and the protein residue. The differences between the amount of Na or of K added in the solutions and the sum of the amounts found in the supernatant and residue were taken to be the amounts present in the original washed protein solution. All four experiments gave the same result (Table IV). On the average the Na/K ratio in the original protein was 3.8, the amounts of Na and K being 5.8 and 1.8%, respectively, of the amounts subsequently added. After equilibration 11.8% of the Na and 8.2% of the K were in the protein residue, the Na/K ratio being 1.46. This result confirms even more clearly the conclusion that this protein, like myosin, prefers Na to K. It is concluded that the published abstract was in error for unknown reasons.

Evidence for a preference of myosin for Na as compared to K is not new. Steinbach (4) found data of this sort in muscle homogenates. Szent-Györgyi(5) on the other hand emphasized the general similarity of K and Na in reactions with myosin although his study of the effects of KCl and NaCl on phosphatase activity of myosin might be taken to indicate slightly greater binding of Na at lower concentrations. (See Fig. 35 p. 54 in first edition 1947). Lewis and Saroff(6) however measured association constants of myosin for Na greater than those for K. J. J. Blum and J. Duke (personal communication) have shown that Na is bound to myosin more strongly than K and when so bound it inhibits the ATP-ase activity of the myosin. Studies of electrophoretic mobility of myosin by Miller *et al.*(7) support the same conclusion. All of these results were obtained with extracted myosin or homogenized muscle. The data reported in this paper are the first in a more or less intact muscle which show the same result.

It is difficult to reconcile all these findings with the theory of Ling(8) which predicts fixed anion sites on myosin with preference for K on account of its smaller effective diameter. Certainly if this is true the myosin must be drastically modified by the process of glycerol extraction or by isolation from the muscle. It seems more likely that destruction of the muscle membrane destroys also the ability to concentrate potassium or to "pump out" the sodium.

Summary. After extraction in 50% glycerol frog muscles retain more sodium than potassium. If they are then immersed in solutions containing equal concentrations of KCl and NaCl the muscles are found to contain again more sodium than potassium. The ability of the normal muscle to concentrate potassium seems therefore to depend upon the intactness of the membrane or the presence of some specific transport mechanism.

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Induction of Allergic Contact Dermatitis in Patients with Sarcoidosis. (23608)

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A diminished incidence of delayed allergic reactions to the intradermal injection of bacterial, viral and fungal antigens is characteristic of sarcoidosis(1,2,3). Since allergic contact dermatitis is also a form of delayed (tuberculin-type) hypersensitivity, similar diminished responses might be expected with contact allergens. The object of the present study was to test this hypothesis using contact sensitizers to which the patient had never been exposed, as well as a commonly encountered contact allergen. By the use of chemical compounds with varying ability to sensitize, some quantitation of the response of patients with sarcoidosis was obtained.

Materials and methods. The sensitizers used in the study were: (1) pentadecyl catechol; (2) 2,4-dinitrochlorobenzene; and (3) paranitrosodimethyl aniline. Hereafter, these compounds will be referred to as PDC, DNCB and NDMA, respectively. PDC is one of the catechols in dermatitis producing plants of the genus Rhus. These catechols are among the most potent sensitizers known, and the incidence of pre-existing sensitization in adults ranges between 75 and 85% when a 1:100 concentration of PDC is used for testing(4). Sensitization to PDC was not attempted, but to test for pre-existing poison ivy sensitivity, 0.25 ml of a 1:100 concentration of PDC in acetone was applied to the forearm of each subject by the open patch method within the area of a cup 2.9 cm in diameter. The test sites were observed after 4 to 6 days. DNCB and NDMA are also potent sensitizers, but it has been found that within a certain range of concentrations, including that used in this study, DNCB is a moderately strong sensitizer and NDMA a weaker one(5,6). In contrast to the observations with PDC in which previous exposure had to be assumed, the subjects were *actively* sensitized with DNCB and NDMA, under controlled conditions of exposure. 0.25 ml of 0.005 molar DNCB and 0.25 ml of 0.005 molar NDMA in acetone were applied to the skin at separate sites by the same open patch method as was used with PDC. Thirty days later the subjects were retested by a similar application at new sites with a 1:1000 dilution of each sensitizer in acetone, and the reactions observed in 4-6 days.

Twenty-three ambulatory out-patients with sarcoidosis, 18 of whom were Negro, were tested with the 3 contact allergens. There were 14 females and 9 males in the group. Although their state of health varied from asymptomatic to chronically ill, none was moribund and all had stigmata of sarcoidosis at the time of the study. One had received 50 mg of cortisone daily for 3 months, an amount not likely to affect sensitization(7).

One hundred thirty-five healthy males of comparable ages, 108 of whom were negro, were used as controls. They were sensitized to DNCB and NDMA and tested to the three compounds in the manner described above. Because Negroes appear to be less easily sensitized to contact allergens as compared to whites (Table I)(4,8), the ratio of Negro to white in the sarcoidosis and control groups was made the same in order to keep the composition of the groups statistically comparable (Table II). No significant sex difference has been observed in the response of normals(9) or patients with sarcoidosis(1) to delayed skin test antigens. Statistical analysis was performed by the chi square method.

Results are summarized in Table II.

1. With PDC, the most potent sensitizer, there was no significant difference in the inci-

TABLE I. Control Group. Comparison of incidence of sensitization to contact allergens between Negro and White.

	DNCB	NDMA
Negro	70/114 = 61%	49/108 = 45%
White	26/ 27 = 93%	17/ 27 = 63%

TABLE II. Incidence of Positive Reactors to Contact Allergens; Combined Negro and White Groups.

	Naturally acquired contact sensitivity	Experimentally produced contact sensitivity	
	PDC	DNCB	NDMA
Controls	101/135 = 75%	96/141 = 68%	66/135 = 49%
Patients with sarcoidosis	18/ 22 = 81%	8/ 23 = 35% (P = <.01)	3/ 23 = 13% (P = <.01)

dence of sensitization in patients with sarcoidosis and in control subjects.

2. With DNCB and NDMA, a highly significant decrease in frequency of sensitization was observed in patients with sarcoidosis as compared to controls. DNCB was about ½ as effective in sensitizing the patients with sarcoidosis as the control subjects, while NDMA, the least potent allergen used, was about ¼ as effective.

Comment. These findings demonstrate that the lowered incidence of reactivity in sarcoidosis is not apparent when a very strong sensitizer, PDC, is used. It is only with weaker allergens that the difference becomes apparent. Actually DNCB and NDMA are considerably more potent than commonly encountered contact allergens such as formalin, vioform and heavy metals(10), so that one might expect a greatly depressed incidence of contact sensitization in patients with sarcoidosis if these latter compounds were to be tested.

Patients with sarcoidosis *are capable* of reacting to these and other skin test antigens although the incidence of sensitization is decreased. For this reason the use of batteries of such tests probably has little diagnostic value in sarcoidosis as has been suggested (11).

Summary. 1) The incidence of naturally occurring skin sensitization to a poison ivy allergen, PDC, and of experimentally induced skin sensitization to 2 chemical sensitizers, DNCB and NDMA, was determined in 23 patients with sarcoidosis and 135 control subjects. 2) The incidence of sensitization to the strongest sensitizer, PDC, was the same in the control group and in the patients with sarcoidosis. 3) With the less effective sensitizers, DNCB and NDMA, a significantly decreased frequency of sensitization was observed in the patients with sarcoidosis. 4) This finding with the latter compounds is consonant with the decreased incidence of delayed type hypersensitivity reactions observed by others using antigens injected intradermally.

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Hemagglutinins Associated with Certain Human Enteric Viruses.* (23609)

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The association of hemagglutinins (HA) with viruses has increased continuously since the classical work with influenza virus(1,2) so that now, in addition to the myxoviruses, HA are associated with the pox viruses, the arbor viruses and, recently, with certain simian viruses recoverable from monkey kidney cell cultures(3). Study of the HA phenomenon in the case of the myxoviruses has led to new concepts of virus-cell relations and in all cases the phenomenon has had immediate or potential practical value as the basis for an *in vitro* method of viral antigen assay or of serologic work. Hence, the new demonstration of HA in relation to a virus group is of possible interest from both the fundamental and the practical standpoints.

This communication will describe in preliminary fashion hemagglutinating properties discovered in relation to several members of the ECHO and Coxsackie groups of viruses. This discovery resulted from efforts to investigate the possibility that certain enteric viruses possessed the ability to agglutinate trypsin-treated red cells similar to that recently reported for the newly isolated respiratory virus, 2060(4); in the course of these efforts it was observed that untreated human erythrocytes of Group O, included for control purposes, were agglutinated by a number of the viral agents tested.

Methods. Hemagglutination. Blood was collected from Group O donors in acid-citrate-dextrose and stored in the refrigerator for no more than a week prior to use. Red cells were washed 3 times with veronal-saline buffer set at pH 7.3 and resuspended finally in 1% concentration. Hemagglutinin (HA) titrations were performed in Kahn tubes by the addition of 0.1 ml of cell suspension to 0.4 ml of serial 2-fold dilutions of virus preparations made in veronal-saline buffer. Readings were by the pattern method of Salk after standing at room temperature for 90 minutes, or until patterns were evident in control tubes (more rapid at 37°C, slower at 4°C). Complete or almost complete ($^{+++}$ or $^{++++}$) agglutination was taken as the endpoint. One HA unit is defined as 0.4 ml of the highest dilution which gives complete or almost complete hemagglutination. *Hemagglutination-Inhibition.* Hemagglutination - inhibition (HI) tests were performed by mixing 0.2 ml aliquots of serum or two-fold serum dilutions with 0.2 ml of viral antigen containing 4 HA units and incubating the mixture at room temperature for 2 hours. After serum-virus incubation, 0.1 ml of cell suspension was added to each tube and the occurrence of HA was observed as described above. Endpoints were taken as the serum dilution completely or almost completely preventing agglutination (0 or $^{+}$). Some animal sera, notably rabbit, possess agglutinins for human Group O

TABLE I. Hemagglutination of Enteric Viruses in Veronal Buffer, pH 7.3, with Human Group O Erythrocytes.

Virus*		HA titer (1:x) at indicated temp. of incubation 8°C	25°C	37°C
ECHO	3	128	64	<2
	4	2	2	2
	6	64	32	<2
	7	2048	2048	2048
	10	32	32	32
	11	256	256	<16
	12	1024	1024	2048
Coxsackie	B_1	$^{+}$undil.	$^{+}$undil.	0
	B_2	$^{++}$undil.	$^{+}$undil.	0
	B_3	32	32	<2†
	B_4	1	1	1
	B_5	$^{++}$undil.	$^{++}$undil.	$^{++}$undil.

* Also tested at all 3 temperatures but not observed to possess HA activity were preparations of polioviruses 1, 2 and 3, ECHO viruses 1, 2, 5, 8, 9, 13 and 14, and Coxsackie A_9.

† After only 60 min. at 37°C, the Coxsackie B_3 HA titer was usually identical with that at 8°C and 25°C but between 60 and 90 min. the HA pattern usually reversed.

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TABLE II. Hemagglutination Inhibition Titers of Rabbit Hyperimmune Sera.

Virus ↓	Serum →	Reciprocal of H-I titer — ECHO 3	6	7	10	11	12	Cox. B_3	ECHO 4
ECHO	3	*4096*	<64	<128	<64	<64	<32	<32	<32
	6	<64	*2048*	<64	"	"	<64	64	<64
	7	"	<64	*1024*	<16	<32	<16	<16	<32
	10	"	"	<32	*4096*	"	"	"	"
	11	"	"	"	<16	*256*	32	"	"
	12	"	"	"	"	<32	*256*	"	"
Cox.	B_3	"	"	<16	<64	<64	<16	*4096*	"

erythrocytes to a titer as high as 1:128. In such cases prior absorption of the serum with 5-10% red cells serves to remove the activity.

Viruses. The stock viruses used here were all prepared in large bottles containing primary outgrowths of trypsinized monkey kidney cells prepared by the usual technics(5). The growth medium consisted of 0.5% lactalbumin hydrolysate and 2% calf serum in Hanks' salt solution, and the confluent sheets of cells were maintained on 0.5% lactalbumin hydrolysate in Hanks' solution.

Results. Virus stocks prepared in monkey kidney cultures were tested for agglutinin activity against Group O erythrocytes at 3 different temperatures as seen in Table I. ECHO viruses 3, 6, 7, 10, 11, and 12 and Coxsackie B_3 exhibited HA activity in dilutions varying from 1:32 to 1:2048. Although significant differences in HA titer were not observed as between reactions carried out at 8°C and at 25°C, the titers obtained at 37°C permitted the clear division of these 7 agents into 2 groups: one (ECHO viruses 7, 10 and 12) with titer unaffected and the other (ECHO viruses 3, 6, and 11 and Coxsackie B_3) with titer greatly depressed. Polioviruses 1, 2 and 3, ECHO viruses 1, 2, 5, 8, 9, 13 and 14 and Coxsackie A_9 were tested at all 3 temperatures but were not observed to possess HA activity. Coxsackie B_1, B_2, B_4, and B_5 and ECHO 4 have exhibited slight HA activity, but since it has not been possible to obtain more potent preparations than those illustrated, further work with these agents has been deferred.

Numerous preparations of the prototype strains of ECHO viruses 3, 6, 7, 10, 11, and 12 and Coxsackie B_3 have been observed to have hemagglutinating activity for human Group O erythrocytes. During the course of these studies, cultures from at least 20 batches of monkey kidney tissue were used, each produced by pooling the kidneys from 3 monkeys. Throughout this time, control culture fluids have never exhibited HA activity. All of 6 Group O donors, including one known Rh negative individual, yielded red cells of comparable activity.

In Tables II and III are summarized the results of HI tests using hyperimmune rabbit sera prepared in this laboratory by Mr. Louis Potash, and monkey sera obtained through the courtesy of Dr. Herbert Wenner. It is to be noted that homologous titers invariably exceeded the heterologous to a significant de-

TABLE III. Hemagglutination Inhibition Titers of Monkey Hyperimmune Sera.

Virus ↓	Serum →	Reciprocal of H-I titer — ECHO 3	6	7	10	11	12	4
ECHO	3	*>16,384*	64	128	128	128	64	64
	6	64	*16,384*	"	64	"	128	256
	7	256	128	*4096*	128	"	64	"
	10	128	64	128	*>16,384*	"	32	64
	11	32	128	"	128	*4096*	"	"
	12	64	32	64	64	32	*4096*	"
Cox.	B_3	128	512*	128	128	256	256	512†

* Pre-immunization H-I titer 1:1024. † Pre-immunization H-I titer 1:128.

TABLE IV. Hemagglutination Inhibition Titers of ECHO Antisera from Different Sources.

	Reciprocal of H-I titer				
Serum → / Virus ↓	Rabbit ECHO 7*	Rabbit ECHO 10*	Rabbit ECHO 11*	Rabbit ECHO 6†	Monkey ECHO 7‡
ECHO 7	*>1440*		40		*>1440*
10		*>1440*			40
11	<20	<20	*160*		
6				*512*	

* Courtesy of Dr. Albert Sabin.
† " " Dr. David Karzon.
‡ " " Dr. Joseph Melnick.

gree.

HI tests using an anti-E7 monkey serum obtained from Dr. Joseph Melnick, anti-E7, E10 and E11 rabbit sera from Dr. Albert Sabin and anti-E6 rabbit serum from Dr. David Karzon yielded similar results (Table IV). Anti-Coxsackie sera (A_9, B_1, B_3, and B_4), prepared by inoculation of mouse passage material into hamsters, were obtained through the courtesy of Dr. Gilbert Dalldorf. When these were tested with B_3 viral HA, the homologous HI titer (1:4096) proved identical with that of the rabbit antiserum prepared by immunization with virus propagated in monkey kidney cultures and far in excess of the maximum heterologous titer (1:128 with B_4 antiserum) observed.

Fifty-five newly isolated strains including 1 of E10, 7 of E11, 12 of E7, 8 of E12 and 27 of E6 provided by Dr. Wolf Henigst and Mr. Louis Potash of this laboratory have been tested with the uniform finding of hemagglutinating activity, either immediately or after a few passages in monkey kidney cultures. The HA activity of selected examples of the above strains of ECHO 6, 7, 10, 11 and 12 has been shown to be specifically inhibited by immune rabbit serum.

Preliminary data indicate that the hemagglutinins reported here are sedimentable by ultracentrifugation and are removed from the supernatant during the process of agglutination in parallel with infectious virus. They are active over a wide range of pH, yielding a fairly constant HA titer in the range of 7.0 to 8.0. Preliminary studies of the hemagglutinin-erythrocyte reaction indicate that hemagglutinin can be eluted from the red cell and that the latter may be so altered as to be inagglutinable by homologous virus. Such virus-treated erythrocytes appear to exhibit a receptor gradient roughly in the order of E6, Coxsackie B_3, E3, E11, E7, E12, and E10. Despite analogies between these findings and some of the characteristics of the myxovirus group, treatment of erythrocytes with Receptor Destroying Enzyme (R D E) or influenza B virus (Lee) appears to result in no decrease of their reactivity with the enteric hemagglutinins and, conversely, enteric virus-treated erythrocytes appear to remain fully reactive with influenza A′ virus (Malaya).

HI titrations of paired human sera collected in relation to infection with Coxsackie B_3 and ECHO viruses 6, 7, 10, 11 and 12 have been performed with the frequent demonstration of a rise in HI titer in the later specimen. The phenomenon of non-specific inhibition, however, has been encountered in both human and animal sera. Until a reliable method is found for removing these inhibitors, the results of HI tests must be interpreted with care.

Discussion. The knowledge that so-called simian viruses are frequently found as contaminants in cultures of monkey kidney cells and that some of these agents possess the ability to agglutinate human Group O erythrocytes(3) should lead to a healthy skepticism when the phenomenon is noted in preparations of enteric viruses grown in this tissue culture system. Indeed, since virus stocks may be contaminated by simian agents, antisera prepared by hyperimmunization of animals with such stocks may conceivably contain HI activity for the contaminant.

The following evidence is available to indicate that the hemagglutinins described in this report are specifically related to the enteric agents, rather than to an hypothetical contaminant. 1) Newly isolated strains of ECHO viruses 6, 7, 10, 11 and 12 have been observed to have HA activity which is specifically inhibited by homologous hyperimmune sera. 2) Hyperimmune sera prepared in 4 different laboratories were found to have HI activity. In the case of Coxsackie B_3, antiserum prepared with mouse passage material was fully as active as that prepared by the inoculation

of virus propagated in monkey kidney cultures. 3) Attempts have been unsuccessful to separate a hypothetical simian contaminant by adaptation of these agents to HeLa, KB and propagable amnion cells (to be reported later) and serial passage in these cultures. 4) Humans infected with these enteric agents (except for ECHO 3 and 10) have been studied serologically, and a substantial number have shown significant rises in HI antibody titers in the post-infection specimens.

Separation of the viruses into 2 groups according to the influence of 37°C incubation on the HA titer results in an interesting parallelism with the grouping of the ECHO viruses proposed by Hsiung and Melnick(6) on the basis of plaque morphology and the susceptibility of patas and rhesus kidney cells. The proposed ECHO virus group A contains all 3 of the ECHO viruses (3, 6, and 11) with HA titers depressed at 37°C while group B contains 2 (E7 and E12) of the 3 viruses with HA titers unaffected by the temperature of incubation. The third member of this latter group (E10) was not assigned to either group A or group B.

Summary. 1) Monkey kidney cell culture fluids containing ECHO viruses 3, 6, 7, 10, 11 and 12 and Coxsackie B_3 have been shown to be capable of agglutinating human Group O erythrocytes in relatively high dilutions. Evidence has been presented indicating that the hemagglutinating activity is specifically related to these agents, rather than to possible simian viral contaminants. 2) Preliminary findings suggest that the HA property resides in the viral particle, that it is absorbed by erythrocytes during the process of agglutination and may be eluted from them, exhausting red cell receptors during the process. Despite such similarities to the myxoviruses, additional data suggest that these agents do not belong to the myxovirus group. 3) Monkey kidney cell preparations of polioviruses 1, 2, and 3, ECHO viruses 1, 2, 5, 8, 9, 13 and 14 and Coxsackie A_9 failed to agglutinate human Group O cells, whereas preparations of ECHO 4 and Coxsackie B_1, B_2, B_4 and B_5 manifested HA activity in low dilution.

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Effects of a Quaternary Derivative of Atropine, N-Benzyl Atropinium Chloride. (23610)

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We have shown(1) that N-benzyl and N-phenacyl atropinium salts, as well as certain other compounds containing quaternary nitrogen atoms, are capable of overcoming the Sarin-induced decrease in twitch response of the indirectly stimulated gastrocnemius-soleus-tibialis anticus muscle group of the cat. The present work was undertaken to study some of the functional changes induced by these compounds.

Methods. Cats, rabbits and guinea pigs of unselected sex were used. Young, male mice weighing about 20 g were used in toxicity determinations. LD_{50} values were calculated by the method of Miller and Tainter(2). Cats and rabbits were anesthetized with sodium pentobarbital (30 mg/kg i.p. or i.v., respectively). A metal bellows manometer recorded blood pressure; intratracheal pressure was recorded from a side-arm cannula by a Marey tambour. ECG was recorded with a Sanborn Viso-Cardiette and EEG by a Grass Electro-

encephalograph. Gut activity was registered through a float actuated by a water-filled balloon in the duodenum. Atropinium salts were dissolved in physiological saline to make 1% solutions. Animals given repeated i.v. doses of N-benzyl atropinium chloride (NBA) received 0.5 to 10.0 mg/kg at 5 to 15-min intervals; before and after each dose they were tested for response to (a) central and peripheral vagal stimulations, (b) occlusion of carotid arteries, (c) i.v. injection of 3 μg/kg of acetylcholine bromide, and i.v. injection of 3 μg/kg of epinephrine hydrochloride. Anesthetized rabbits were used for kymographic recording of the contractions of the nictitating membrane, excited indirectly by square-wave stimuli (frequency 50/sec., pulse duration 0.1 msec., strength 10 v.) applied to the preganglionic fibers of the superior cervical ganglion. Isolated rabbit duodenum or frog rectus abdominis muscle was suspended in oxygenated Ringer-Locke solution or aerated Ringer solution, respectively. The strips were exposed to concentrations of NBA of from 0.1 to 100 mg/100 ml. Contractions were recorded kymographically. The response of the gastrocnemius-soleus-tibialis anticus muscle group of the cat was recorded as in our previous paper(1).

Results. NBA had an i.p. LD_{50} for the mouse of 62.5 $\pm$ 3.0 mg/kg and for the guinea pig of 105 $\pm$ 5 mg/kg. By rapid i.v. injection NBA had an LD_{50} for the mouse of 7.4 $\pm$ 0.2 mg/kg and N-phenacyl atropinium bromide (NPA) had one of 9.7 $\pm$ 1.5 mg/kg. For both compounds speed of injection by the i.v. route had a marked effect on toxicity. Thus, 15 mg/kg of NBA could be given i.v. to mice without fatality if spread over 5 min. For comparison, the i.p. LD_{50} of atropine sulfate for the mouse is 223 mg/kg and for the guinea pig is 200 mg/kg; the i.v. LD_{50} for the mouse is 75 mg/kg and is only slightly dependent on the rate of injection. Because the hypotensive effect of NPA also was greater than that of NBA and because we sought a therapeutic compound without too marked side effects, our study was confined then to NBA.

I.v. injection of 4 mg/kg of NBA into an anesthetized cat not only lowers blood pressure but also slows the rate of respiration as well as decreasing the tidal volume. To determine whether these effects are central or peripheral in origin, 2 cats were given intracarotid or intrajugular injections of 1 mg/kg of NBA. The results are summarized in Table I, which shows that intrajugular injection of NBA produced almost twice as much slowing of respiration and very nearly the same hypotension as intracarotid injection. It seems likely, therefore, that NBA exerts its effects on respiration and blood pressure through peripheral rather than central actions. This conclusion is supported for blood pressure by findings that NBA-induced hypotension cannot be prevented by prior atropinization, by bilateral vagotomy or by section of the cervical spinal cord with bilateral vagotomy. NBA (4 mg/kg i.v.) blocked responses of blood pressure and of duodenum to both central and peripheral vagal stimulations, to occlusion of the carotid arteries and to injected acetylcholine. It had no significant effect on responses to injection of epinephrine. Central vagal stimulation was still capable of slowing respiration although it had no effect on blood pressure. These findings suggest again that the principal actions of NBA are on autonomic effectors rather than upon their centers of innervation.

TABLE I. Effects of 1 mg/kg of N-Benzyl Atropinium Chloride, Injected into Carotid Artery or Jugular Vein, on Blood Pressure and Respiration in the Cat Anesthetized with Sodium Pentobarbital.

Drug effect	Intracarotid		Intrajugular	
	Cat 1	Cat 2	Cat 1	Cat 2
	%			
Slowed respiration	17	7	24	21
Depressed blood pressure	74	70	64	68

Duodenal activity was depressed by small doses (1 mg/kg) of NBA; larger doses (2 to 5 mg/kg) increased both intestinal tonus and motility. *In vitro,* NBA (0.1 to 100 mg/100 ml) increased the tonus of rabbit duodenal segments, this effect being blocked partially by atropine. NBA (0.2 mg/100 ml) blocked the stimulant action of acetylcholine (0.2 mg/100 ml) on isolated duodenum or relaxed the segment when applied after acetylcholine. In

both cats and rabbits, NBA (1 to 10 mg/kg) produced no striking alterations of the ECG or EEG patterns. Low doses produced an initial tachycardia, followed by definite slowing of the heart rate. Higher doses produced immediate slowing of the heart and a general lowering of the potential of the EEG record, with the appearance of occasional alpha spindles.

Although NBA appears to have no significant effect on central structures, a dose of 4 mg/kg lowers by 60% the isotonic contraction of the nictitating membrane in response to electric stimulation of the preganglionic fibers of the superior cervical ganglion. The response returns to normal within about 20 min after injection of NBA. Atropine sulfate (5 mg/kg i.v.) produced no more than 10% depression of the same response, lasting for less than 5 min. It is apparent, therefore, that NBA is much more potent in decreasing transmission of excitation from preganglionic fibers to the effector than is atropine itself.

Experiments with isolated frog rectus abdominis *in vitro* showed that atropine sulfate in a concentration of 5 mg/100 ml produced only a slight, temporary block of stimulation by acetylcholine although NBA in a concentration of 0.5 mg/100 ml blocked completely stimulation by acetylcholine. Similarly, in the anesthetized cat NBA (5 mg/kg i.v.) blocked the stimulatory effect of acetylcholine (50 μg/kg), injected intraarterially, on skeletal muscle. About 30 min. after injection of NBA are required for the response to acetylcholine to return to normal.

Discussion. It is apparent that NBA has some of the antimuscarinic properties of atropine: ability to block the effects of vagal stimulation on blood pressure and heart rate and ability to block the effects of acetylcholine on blood pressure, heart rate and duodenal tonus. Shea(3) has shown that NBA is about ¼ as active as atropine sulfate in inhibiting secretion by the rat's stomach. In addition, NBA has certain effects not elicited significantly by atropine: fairly marked lowering of blood pressure, block of transmission of excitation from preganglionic fiber to nictitating membrane, block of stimulation of muscle by acetylcholine and acceleration of recovery from Sarin-induced block of responsiveness of muscle to electrical stimulation of its motor nerve. NPA is similar to NBA, so far as we have studied it, but has a more pronounced hypotensive action and a greater over-all lethality.

The stimulation of the smooth muscle of the gut by NBA, because it occurs in isolated segments of gut, must be a direct effect of the chemical on the peripheral structure. The effects on heart rate, blood pressure and respiration also seem to depend in large part, at least, on peripheral actions. This is in accord with the observation that, in general, quaternary compounds do not enter the central nervous system readily.

If NBA has a modality of activity not possessed by atropine, it should be adjunctive to the latter drug in the prevention of mortality from administration of Sarin. In rabbits given both Sarin and atropine intravenously, 2 mg/kg of atropine sulfate protected 3 out of 6 animals given 30 μg/kg of Sarin; and 0 out of 6 animals given 60 μg/kg of Sarin; 5 mg/kg of atropine sulfate protected 3 out of 6 rabbits given 30 μg/kg of Sarin and 1 out of 6 given 60 μg/kg of Sarin. A mixture of 2 mg/kg of NBA and 2 mg/kg of atropine sulfate protected 4 out of 6 rabbits given 30 μg/kg of Sarin and 2 out of 6 given 60 μg/kg of Sarin. NBA appears, therefore, to be adjunctive to atropine, at least in the rabbit.

Summary. Quaternization of atropine by the benzyl or phenacyl group* increases the lethality of the compound rather markedly; the resultant compounds have the antimuscarinic properties of their parent but to a lesser degree. They possess, in addition, a hypopneic and a potent, but brief, hypotensive action. The latter is especially marked in N-phenacyl atropinium bromide; it is not mediated through the carotid sinus or carotid body mechanisms or the vagal centers, but is largely peripheral in origin. N-benzyl atropinium chloride (NBA) prevents stimulation of striated muscle by acetylcholine and prevents transmission of excitation to the nictitating membrane from preganglionic fibers of the superior cervical ganglion; NBA has,

* NBA and NPA were made by Mr. Lawrence J. Edberg of the Directorate of Medical Research.

therefore, antinicotinic properties which are lacking almost entirely in atropine.

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Amino Aciduria in Primates Following Irradiation.* (23611)

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The acute radiation syndrome presents a symptom complex that is dose dependent. In the lower lethal dose range for the primate, the metabolic damage is expressed in 2 phases, an initial phase of predominantly alimentary dysfunction and a later phase associated with the catabolism of body tissues. In both phases, nitrogen balance becomes negative, but little change in the total daily urinary nitrogen excretion takes place until as an antemortem event, the daily urine nitrogen excretion may be greater than during a pre-radiation control day.‡ Some qualitative change in the nitrogen partition occurs and amino acid excretion is increased. This increase has been stated to be due to the radiation injury sustained by the animal(1). This paper presents a study of the urine amino acid nitrogen excretion of 4 whole body irradiated monkeys and of the 2 survivors (after physiological recovery) when sham irradiated and given the same dietary intake as in their radiation experiment.

Material and method. Adult male imported Rhesus monkeys (*M. mulatta*), tuberculin tested and separately housed, were placed in a metabolic cage. Their body weights varied from 6.8-12.4 kg and they were in robust health. They were fed by hand on Parkes "41" rat cubes and hand watered, so an excellent estimation of food and water intakes was possible. After a control period, the animals were whole body irradiated or sham irradiated and the urine amino acid nitrogen (amino N) and total nitrogen (total N) excretion followed until death or obvious recovery. The urines, voided into flasks with toluene as a preservative, were collected at the same time each day and analyzed for amino N by the copper titration method(2), and for total N by a semi micro method(3). Several urines were subjected to paper chromatography(4). *Radiation factors.* One animal was exposed to X irradiation, 240 Kv, HVL. 1.2 mm Cu with stepped Cu/Al filter, F.S.D. 150 cm, dose-rate 9 r/min. and the other 3 to gamma radiation (Co^{60}, 1.3, 1.1. Mev.) dose-rates 13-15 and 103-105 r/min. With both the X and gamma irradiation, the radiation procedure was arranged so that the dose received was relatively homogenous throughout the animal (5). Measurement of dose-rate was made with a Victoreen 100 r dose meter at various selected positions that the animal would subsequently assume. Thiopentone anaesthesia was given for the X irradiation(6).

Results. The daily urine amino N excretion of the 4 irradiated animals is shown in Table I. Since amino N is but a part of the total urinary N excretion, total N excretion is recorded as well. The values for the 4 animals during the control period were in close agreement, with the exception of monkey C, a smaller animal.

Immediately following radiation, only monkey C showed an amino N excretion greater

* Experiments carried out at the M.R.C. Radiobiological Research Unit, A.E.R.E., Harwell, England.

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‡ Unpublished observations, Hunter, C. G.

TABLE I. Daily Urine Amino N and Total N (in Parentheses) Excretion, g/day, in 4 Monkeys after a Single Exposure. Animal G—two exposures to whole body X and gamma irradiation in lower lethal dose range for the primate.

	Non-survivors		Survivors	
Monkey	C	G	A	E
Dose	500 r x	460 r γ	480 r γ	480 r γ
Pre-irrad. (No. days)	6	5	5	4
Mean	.02 (1.98)	.092 (4.37)	.094 (4.42)	.078 (2.94)
Range	.017–.022 (1.52–2.48)	.064–.124 (4.142–4.645)	.079–.113 (4.24–4.52)	.072–.084 (2.64–3.23)
Days post-irrad.				
1	.038 (2.54)	.08 (2.90)	.079 (3.60)	.072 (2.17)
2	.031 (1.10)	.076 (2.88)	.097 (3.89)	.08 (2.10)
3	.03 (2.58)	.076 (2.60)	.104 (2.88)	.089 (1.70)
4	.034 (2.16)	.072 (2.58)	.113 (2.87)	.079 (1.23)
5	.022 (2.37)	.088 (2.26)	.113 (2.87)	.075 (1.15)
6	.028 (3.48)	.065 (2.28)	.087 (2.27)	.084 (1.99)
7	.08 (3.90)	.088 (2.04)	.085 (2.13)	.111 (1.109)
8	.08 (3.81)	.119* (3.31)	.064 (1.49)	.104 (1.15)
9	.053 (1.76)	.16 (3.20)	.083 (1.898)	.112 (1.65)
10	.042 (3.28)	.163 (3.88)	.083 (3.20)	.123 (1.58)
11		.098 (2.55)	.099 (2.48)	.154 (2.40)
12		.103 (3.14)	.098 (2.49)	.133 (2.06)
13			.089 (2.80)	.154 (2.62)
14			.088 (2.79)	.10 (3.31)
15			.094 (2.52)	.065 (3.22)
16			.072 (3.40)	.077 (3.70)

* Day 1 after 2nd irradiation.

than in the control period. By the end of the first week all values were within the pre-irradiation range, but the amino acid index $\left(\frac{\text{amino N}}{\text{total N}} \times 100\right)$ had increased slightly. With the onset of severe terminal catabolism in animal C and animal G (after the second exposure), the amino N values were higher than during the control period. One survivor only, animal E, excreted significantly more amino N than before irradiation and this occurred during N retention at the end of the second week.

Table II records the results of the sham irradiation experiment. The mean control values of both animals were slightly higher, perhaps associated with an increase in weight since irradiation (12 months previously). With the identical dietary intake as in their irradiation study, monkey A lost more amino N and total N than after irradiation. Monkey E on the other hand, gave an opposite result losing more amino N and total N after irradiation.

Two dimensional chromatograms were run on the urine of monkey A. Normal monkey urines were found to contain 2 amino acid groupings: glutamic acid/glycine and taurine/glutamine in descending order. After irradiation slight quantitative increase was demonstrated on days 4 and 5, but the only

TABLE II. Daily Urine Amino N and Total N (in parentheses) Excretion, g/day, in 2 Monkeys Sham Irradiated and Given the Same Dietary Intakes of Their Irradiation Experiment.

	Monkey A	Monkey E
Days pre-sham irrad.	3	3
Mean	.112 (3.77)	.12 (3.61)
Range	.104–.12 (3.46–4.10)	.103–.154 (3.36–4.00)
Days post-sham irrad.		
1	.112 (3.28)	.076 (3.80)
2	.09 (2.80)	.078 (2.23)
3	.091 (2.80)	.068 (1.94)
4	.112 (3.62)	.065 (2.82)
5	.114 (3.42)	.073 (2.21)
6	.084 (2.10)	.074 (1.65)
7	.057 (3.68)	.067 (1.55)
8	.096 (3.06)	.076 (2.82)
9	.104 (2.65)	.053 (2.42)
10	.122 (3.20)	.06 (2.503)
11	.122 (3.20)	.072 (2.78)
12	.126 (3.88)	
13	.127 (4.56)	
14	.13 (4.84)	

qualitative difference was the appearance of small amounts of leucine for 9-10 days.

Discussion. Amino aciduria has been described in man following accidental over-exposure to ionising radiation(1,7). Two lethally irradiated men excreted increased quantities of urine amino acids during the first week, the peak excretion being on the sixth day, when values about 3 times the normal were excreted. Three other males and one female, much less severely irradiated, also showed increases during the first week. In these reports urine amino acid excretion was estimated by paper chromatography and the pattern of the amino acids changed from the four acids, glycine, taurine, serine and alanine, excreted in normal human urine to some 14-15 in the irradiated. In spite of the paucity of data given in these reports, radiation injury appeared to increase urinary amino acid excretion, the cause for this increase not being determined. It was obvious that the increase appeared quickly after the exposure being detected on the first day although the amount excreted was not proportional to the dose received.

Amino aciduria is a sign of hepatic and renal injury, but there is no evidence that radiation injury in doses given in this study affects the function of the liver and kidney (1,4,7). In primate radiation injury it has been suggested that the amino aciduria may be due to failure of peripheral utilization of amino acids(7), but since little difference exists between the amino acid excretion in the irradiated monkey and the monkey fed identically with its irradiated self, dietary intake must be a factor as well. Very little evidence can be adduced that radiation injury *per se* in the lower lethal dose range causes the reported amino aciduria. So the increased values of urinary amino acids should be regarded properly as a reflection of the aberration of protein metabolism usually associated with the response of the healthy primate to fevers, infections and trauma(8,9).

Summary. The daily urine amino N excretion of 4 monkeys was followed before and after whole body irradiation with doses in the lower lethal range. In 2 non-survivors, there was little change in the daily quantity excreted until terminally. In 2 survivors given the same food intakes as in the irradiated study and sham irradiated, the daily urine amino N excretion during the first week differed but slightly from the values after irradiation, but in the second week more amino N was excreted after irradiation in one animal and less in the other. It would appear that amino aciduria in primates irradiated with doses in the lower lethal range is inseparable from the natural response of the overall protein metabolism associated with any injury.

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A Fatal Reaction Caused by Implantation of Adult Parental Spleen Tissue in Irradiated F_1 Mice. (23612)

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The importance of splenic and lymphoid tissue in antibody production, and the ability of these cells to confer immunity when grafted into other animals is well documented(1-5). Billingham, Brent, and Medawar(6) introduced the expression, "adoptive transfer," to describe the transfer of immunity by immunologically activated tissue. Mitchison detected the presence of implanted donor spleen cells in the lymph nodes and spleen of irradiated recipients(7). He also noted that the antibody titer after administration of preimmunized spleen is maintained considerably longer in irradiated than in nonirradiated recipients(8) and that production of antibody is more active in the former(7). The influence of the internal environment of the implanted cells on their transfer of the antibody response is seen in the inability of the neonatal rabbit to produce antibodies after transfer of sensitized adult rabbit lymphoid cells unless normal adult lymphocytes have been injected previously(9,10), whereas in the irradiated adult recipient, neonatal lymphoid cells are effective and preinoculation of normal lymphocytes is not required.

In the investigation of possible applications of these immunogenetic principles in the radiotherapy of mouse leukemia, we attempted to transfer to leukemic animals splenic cells from a tumor-resistant donor. Mice of the (C3H x 101) F_1 strain harboring a serially transferred, indigenous leukemia, were inoculated, after supralethal irradiation, with adult spleen tissue from strain-101 donor mice that had been preimmunized against the leukemia. Unexpectedly, these animals died before the untreated leukemic controls. Subsequently, groups of leukemia-free mice were similarly treated; and they, too, died in the second and third week after irradiation, regardless of whether (C3H x 101) F_1 or parent strain (101) bone marrow was administered concomitantly, with the parent spleen tissue, to enhance recovery of hemopoietic tissues from radiation injury. The fatal reaction to the implantation of parent spleen is the subject of this report.

Methods. Eight- to 16-week-old (C3H x 101) F_1 male mice were exposed to a single LD_{100}/30-day dose of total-body radiation of 800 to 1000 r. The mice were irradiated in a lucite drum divided into 12 compartments separately housing each animal, which was continuously revolved in the treatment field at approximately 7 rpm. The physical factors were 250 kvp, 30 ma, 3 mm of Al added filtration, 0.40 mm of Cu, 82 cm target-to-mouse distance, and dose rate 98 r/minute. The spleens from 5 to 10 adult 101 or (C3H x 101) F_1 mice were pooled, minced, ground, and then suspended in Tyrode's solution by a Teflon homogenizer. The tissue was chilled throughout the procedure. Counts revealed the presence of approximately 100 x 10^6 grossly intact, nucleated cells per spleen. Different groups of (C3H x 101) F_1 mice received the equivalent of one-fourth to one spleen (25 x 10^6 to 100 x 10^6 cells) intraperitoneally 3-5 hours after X-irradiation, the implanted tissue being administered 1 hour after sacrifice of the donor. One femoral equivalent from an isologous or parent strain (101) donor was also injected into each recipient via tail vein on the day after irradiation, except in the controls, which received the bone marrow inoculum alone 3-5 hours after irradiation. Bone marrow cell suspensions in Tyrode's solution were prepared in the manner described by Urso and Congdon(11). The parent (101) spleen and bone marrow donors were 14-20 weeks old; in the earliest experiments they were preimmunized 3-7 weeks before transfer by the intraperitoneal adminis-

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TABLE I. Survival of F_1 Hybrid Mice Injected with Parental Strain Spleen Cells.

Recipient		Implanted spleen		Recipients surviving 30 days		Median time of death
X-ray dose (r)	Strain	Donor strain	No. cells inj. ($\times 10^6$)	No.*	%	(days)
800–1000	(C3H × 101)F_1	101†	75–100	2/42	5	12
"	"	"	25– 50	13/45	29	16
0	"	"	100	10/10	100	
900–1000	"	101‡	100	2/24	17	12
800– 900	"	"	50	43/49	88	19
0	"	"		9/ 9	100	
800–1000	"	(C3H × 101)F_1	50	16/16	100	
400	"	101†	50	8/10	80	19–24
"	(RF × AKR)F_1	RF†	20	0/ 9	0	17
0	"	" †	20	9/ 9	100	
900	(C57BL × 101)F_1	C57BL‡	100	0/20	0	18
"	"	"	50	2/ 7	29	26
"	"	(C57BL × 101)F_1	50–100	20/20§	100	

* No. surviving/No. inj. † Presensitized. ‡ Non-presensitized. § Equal numbers of mice received 50 × 10^6 and 100 × 10^6 cells.

tration of leukemic (C3H x 101) F_1 liver and spleen cell suspensions and in later experiments, by the intravenous injection of normal (C3H x 101) F_1 liver, spleen, and kidney cell suspensions one week before transfer. Additional 8-week-old male (C3H x 101) F_1 and 14- to 15-week-old (RF x AK) F_1 female mice were exposed to sublethal doses of X-radiation (400 r), 3-5 hours and 24 hours, respectively, before they received immunized adult parental spleen tissue intravenously. RF mice that had received normal AK spleen tissue intravenously one week earlier served as preimmunized spleen donors for the (RF x AK) F_1 recipients. Three groups of 12-week-old (C57BL x 101) F_1 female mice exposed to 900 r received nonsensitized adult C57BL spleen cells intravenously. None of the (C3H x 101)F_1 sublethally irradiated, (RF x AKR)F_1 or (C57BL x 101)F_1 mice received bone marrow.

Results. Thirty-day survival. The vast majority of irradiated F_1 mice receiving parental strain spleen, with or without subsequent administration of either F_1 or parental strain bone marrow, died in the second to fourth week after treatment (Table I, Fig. 1). These mice looked well for the first week after inoculation but deteriorated thereafter. Spleen cells of presensitized parental mice caused greater mortality than those of nonsensitized mice, relatively few recipients of the smaller quantities of nonsensitized spleen cells succumbing in the first month (Table I). Of the irradiated F_1 mice (controls) receiving bone marrow without spleen, 19 of 20 recipients of isologous (F_1) and 92 of 98 recipients of parental marrow survived the initial 30 days after exposure. Later deaths among recipients of presensitized parental bone marrow are to be reported in a subsequent communication. Mice receiving parental spleen and bone marrow without prior irradiation and others inoculated with F_1 spleen after X-irradiation suffered no apparent ill effects.

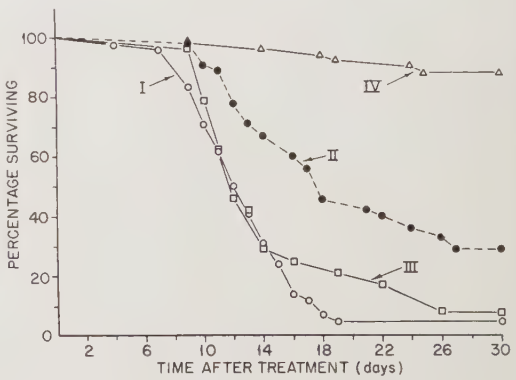

FIG. 1. Survival of irradiated (C3H × 101)F_1 mice inoculated with spleen cells from donor mice of the 101 strain. Curve I, received 75-100 × 10^6 cells from a presensitized donor; curve II, received 25-50 × 10^6 cells from a presensitized donor; curve III, received 100 × 10^6 cells from a non-presensitized donor; curve IV, received 50 × 10^6 cells from a non-presensitized donor.

Each mouse received in addition to spleen cells the equivalent of 1-2 femurs of isologous or 101 strain bone marrow cells, the strain of origin of the marrow having no detectable influence on the 30-day survival.

Autopsy findings. In those mice found dead after exposure to 800-1000 r, there was atrophy of hemopoietic elements in the bone marrow and spleen, with the exception of those in which parental marrow was administered in addition to parental spleen. In the latter, the marrow and red pulp of the spleen were cellular, exhibiting active formation of granulocytes, erythrocytes, and megakaryocytes. The lymphoid follicles were atrophic in all animals, with little or no evidence of lymphopoiesis. Foci of necrosis of varying extent were seen in the liver, and some mice showed extensive bacterial invasion. Focal necrosis of the mucosa of the large intestine, with infection and hemorrhage was also noted. In the sublethally irradiated (RF x AK) F_1 mice, there were, in addition to the previously mentioned findings, reticuloendothelial hyperplasia, with pseudotubercle formation and areas of fibrinoid necrosis in the spleen and lymph nodes.

Discussion. In this investigation, the implantation of adult parental strain spleen cells prevented the survival of irradiated F_1 hybrids, even when the recipients received a sublethal dose of X-radiation (400 r = $LD_0/30$ days) or subsequent inoculation of isologous F_1 hybrid marrow cells. Since this effect was not observed when isologous F_1 hybrid spleen cells were injected, the reaction is believed to have resulted from immunogenetic differences between hybrid and parent tissues. Because tissues of the F_1 hybrid are antigenic to mice of the parent strain but not vice versa, and because of the capacity of transplanted spleen cells to confer "adoptive immunity," we inferred that the fatal reaction described represents the effects of production by the grafted parental spleen cells of antibodies against the tissues of the F_1 recipient. In all experiments, the dose of parent spleen cells was correlated with the median time of death of the lethally irradiated host (Table I). Although relatively few recipients of less than 75 million adult parental spleen cells died within the first 30 days, many others succumbed in the second month. After compilation of these results, Makinodan *et al.*(12) observed a similar fatal reaction in lethally irradiated (C3H x 101) F_1 mice injected with spleen cells obtained from homologous adult mice of the LAF_1 strain. Injection of spleen cells from newborn homologous donors apparently does not produce the early death(13) caused by adult parental strain and adult homologous spleen cells, but the effect of spleen cells from newborn donors of the parental strains has not been tested.

The absence of this reaction in nonirradiated F_1 recipients may have been caused by lack of proliferation of the implanted spleen cells, owing to the normal hemopoietic status of such recipients and to the lack of need for cell proliferation such as is required to repair the radiation injury in the irradiated recipients. Immature animals obviously have a natural stimulus for cell proliferation; thus, situations possibly similar to this one have been reported by Billingham and Brent(14), who noted death in newborn rats after inoculation of homologous antibody-forming cells, and by Simonsen(15), who found hemolytic anemias in newborn chicks inoculated with adult spleen cells prior to hatching.

The pathologic findings and time of death indicate that some of the mice receiving parental spleen died of bone marrow aplasia; in other animals, however, the bone marrow was cellular and the immediate cause of death cannot be given. The granuloma-like reactions in the spleen and lymph nodes of sublethally irradiated F_1 mice receiving immunized parental spleen further suggest that these tissue changes occur as part of, or in response to, an immunologic reaction.

It is interesting that the marrow cavity of mice receiving parental marrow cells and spleen cells was cellular, whereas the marrow of mice receiving *isologous* (F_1) marrow cells along with parental spleen cells, or in that of mice receiving parental spleen cells alone, was usually atrophic. This is in keeping with the interpretation that the grafted spleen cells react against antigens in the F_1 recipient that are inherited from the homologous parent and that, of course, tissue antigenically identical with the graft is unaffected.

Uphoff(16), Koller(17), and Trentin(18) invoked a similar mechanism to account for delayed mortality in irradiated F_1 mice of strains other than those used in this study

after treatment with bone marrow of parental strain donors. It is noteworthy, however, that in the strains used in this investigation, as in the work of van Bekkum and Vos(19), there was little or no delayed mortality in irradiated F_1 recipients of parental bone marrow; *i.e.*, only 11 of 59 irradiated (C3H x 101) F_1 mice given marrow from donors of the 101 strain died within 90 days after irradiation, whereas the injection of spleen cells from 101-strain donors into irradiated (C3H x 101) F_1 recipients caused extensive and prompt mortality, as described. These observations suggest that factors other than the histoincompatibility of the parental strains influence the probability of the occurrence of delayed reactions in F_1 recipients of parental marrow. One such factor is the immunologic status of the bone marrow donor, since on injection of marrow alone from donors of the 101 strain presensitized to (C3H x 101) F_1 tissues, there was appreciable delayed mortality of irradiated (C3H x 101) F_1 recipients (20); as noted, this effect is not observed with marrow from nonpresensitized 101-strain donors. It seems, therefore, that in the pathogenesis of the delayed parent bone marrow reaction, as in the parent spleen reaction, the number, type, rate of growth, and immunologic status of the implanted cells affect the severity of the reaction, as does the relative degree of immunogenetic incompatibility of the donor and recipient(21,16).

Summary. 1) Implantation into irradiated F_1 hybrids of viable spleen cells from nonirradiated adult mice of the parental strains caused death within 2-4 weeks. 2) The fatal reaction occurred even after sublethal doses of radiation or when the recipients were also injected with marrow cells from isologous hybrids or from mice of the same strain as that from which the parent-strain spleen tissue was obtained. The deaths are ascribed to the formation by the implanted cells of antibodies against the recipient.

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Pyruvate and Glucose as Precursors of Acetoacetate. (23613)

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Although carbohydrate is known to be "antiketogenic," pyruvate has been shown to form acetoacetate in cell-free liver preparations(1,2,3). In the present series of studies, respiring homogenates of lactating guinea pig mammary gland were found to convert pyruvate and acetate to acetoacetate at rapid rates, in contrast with a slow, but measurable rate of conversion of glucose to acetoacetate(4).

The antiketogenicity of carbohydrate has been attributed to its ability to give rise to oxaloacetate, an explanation which is not accepted as entirely satisfactory(5). Since ketogenesis is an alternative to lipogenesis(6), and the latter is a synthetic process requiring the participation of reduced pyridine nucleotide(7), the possibility was considered that the ability of glucose to regenerate reduced pyridine nucleotide in respiring systems might be a key factor in determining its low ketogenicity. This hypothesis could be tested by studying the behavior of lactate, a metabolite closely related to pyruvate, but whose oxidation, like the breakdown of glucose, is coupled with the reduction of DPN.

Methods. Homogenates of mammary glands of lactating guinea pigs, prepared as described in a previous study of lipogenesis (8), and rabbit kidney cortex homogenates were incubated in a reaction mixture containing in addition to KCl: $MgCl_2$, 0.01 M; nicotinamide, 0.02 M; DPN, 3×10^{-4} M; ATP, 0.001 M; phosphate buffer, pH 7.4, 0.01 M aminotrishydroxymethylmethane (tris) buffer pH 7.4, 0.01 M and carrier acetoacetate, 0.01 M. After incubation with C^{14}-labeled substrates (0.005-0.01 M) in the Warburg apparatus, the reaction mixtures were acidified and the bound CO_2 collected in the NaOH in the center wells which had served to absorb the CO_2 during the measurements of O_2 consumption. The contents of the center wells were then removed, replaced by fresh NaOH and the acetoacetate was decarboxylated by the addition of aniline citrate. After addition of carrier K_2CO_3 the samples containing the respiratory CO_2 and the terminal CO_2 of acetoacetate respectively were precipitated with $BaCl_2$. Thin samples of $BaCO_3$ were mounted on planchets of 4.9 cm^2 area and counted with a gas-flow counter with 'Micromil' window. After correction for self-absorption, the 'total counts' were calculated and converted to mμmole-equivalents of the radioactive substrate oxidized. Although the radioactivity of only the terminal carboxyl group of acetoacetate was determined, it was assumed that both halves of the molecule were equally labelled and the radioactive acetoacetate was calculated as being derived from two molecules of pyruvate, lactate or acetate and from one molecule of randomly labeled glucose respectively.

Results. Experimental data representative of mammary and of kidney homogenates are shown in Table I. The high rates of conversion to acetoacetate of pyruvate and acetate are in contrast with the low ketogenicity of glucose and lactate. The addition of hexokinase, which accelerated the rate of aerobic glycolysis and increased the amount of glucose oxidized to CO_2, had no effect on the rate of its conversion to acetoacetate.

Since the oxidation of lactate differs from that of pyruvate in only one additional oxidative step coupled with the reduction of DPN, it may be concluded that the ability to regenerate reduced pyridine nucleotide, shared by glucose and lactate, is a factor of major importance in determining their low ketogenicity. In mammary homogenates, incubated with acetate or pyruvate, increasing amounts of fumarate have been found to cause progressive stimulation of lipogenesis(8) and progressively to depress acetoacetate formation (4). It seems probable that the effect of fumarate in large quantities, exceeding the small amounts required to catalyze Krebs cycle oxidations, is also due to its power to maintain pyridine nucleotides in the reduced form. Acetoacetyl-coenzyme A is obviously formed from pyruvate, and must also be formed from

TABLE I. Formation of Acetoacetate from Pyruvate, Lactate, Glucose and Acetate.

Exp. 1—Guinea pig mammary gland homogenate, dry wt 15.8 mg/vessel; incubation period 80 min.

Exp. 2—Rabbit kidney cortex homogenate (centrifuged to remove cell debris and nuclei), dry wt 19.8 mg/vessel; incubation period, 50 min.

Exp. No.	Additions	$-Qo_2$	Labeled substrate (acetyl-coenzyme A equivalents), mμmoles/100 mg dry wt/hr, appearing in: Respiratory CO_2	Labeled substrate (acetyl-coenzyme A equivalents), mμmoles/100 mg dry wt/hr, appearing in: Aceto-acetate
1	2-C^{14}-pyruvate, 0.005 M	8.3	3010	5320
	2-C^{14}-DL-lactate, 0.005 M	5.9	3580	812
	C^{14}-glucose (r.l.), 0.005 M	8.8	6400	700
	Idem and hexokinase, 18 units	10.2	9000	780
2	2-C^{14}-pyruvate, 0.005 M	15.9	4770	11160
	2-C^{14}-DL-lactate, 0.01 M	17.0	7920	1430
	C^{14}-glucose (r.l.), 0.005 M	20.0	5820	2090
	1-C^{14}-acetate, 0.005 M	17.6	8620	9680

lactate and from glucose. In the presence of reduced pyridine nucleotides, as during the oxidation of the weakly ketogenic glucose or lactate, or of the ketogenic substrates supplemented with fumarate, acetoacetyl-coenzyme A appears to be reduced to β-hydroxybutyryl-coenzyme A, initiating the chain of reactions in the reversal of the fatty acid cycle(7). In the case of the ketogenic substrates undergoing oxidations in the absence of a source of reduced pyridine nucleotide, enzymic deacylation of acetoacetyl-coenzyme A would result in the formation of acetoacetate.

The ability of glucose to regenerate reduced pyridine nucleotide in an aerobic system, may explain not only its non-ketogenicity, but also its effectiveness in stimulating the synthesis of fatty acids from acetate, originally demonstrated in mammary slices(9) and later observed in actively respiring mammary homogenates metabolizing acetate in the presence of fumarate(8).

Whereas C^{14}-labeled glucose (randomly labeled), when present as the only precursor of acetyl coenzyme A, was virtually non-ketogenic, unlabeled glucose, when added to mammary homogenates converting 1-C^{14}-acetate to acetoacetate, showed no antiketogenic activity. In some preparations in which the rate of acetoacetate formation from acetate was relatively slow, glucose even accelerated the rate of incorporation of acetate carbon into acetoacetate (Table II). The 'ketogenic' effect of glucose persisted in the presence of fumarate in catalytic amounts, but was abolished when the concentration of fumarate was increased.

The increased rate of $C^{14}O_2$ production

TABLE II. Effect of Glucose on Formation of Acetoacetate from Acetate Guinea Pig Mammary Gland Homogenates. Exp. 1: dry wt 23.5 mg/vessel; incubation period 85 min. Exp. 2: dry wt 59 mg/vessel; incubation period 60 min. All flasks contained 1-C^{14}-acetate, 0.005 M.

Exp. No.	Additions	$-Qo_2$	Acetate (mμmoles/100 mg dry wt/hr) appearing in: Respiratory CO_2	Acetate (mμmoles/100 mg dry wt/hr) appearing in: Aceto-acetate
1	None	4.0	2340	262
	Glucose, 0.02 M	8.0	3660	1520
	Fumarate, 0.001 M	7.5	3760	274
	Idem; glucose, 0.02 M	11.0	3660	1130
	Fumarate, 0.002 M	7.8	2140	156
	Idem; glucose, 0.02 M	9.2	1870	223
2	None	6.5	3650	1825
	Glucose, 0.005 M	7.3	2900	2670
	Idem; hexokinase, 18 units	6.6	2540	1615

from 1-C^{14}-acetate, observed on addition of glucose, indicates that the rate of activation of acetate was accelerated by the breakdown of glucose. It may be assumed that under the experimental conditions employed, the rate of formation of acetoacetyl-coenzyme A from the acetyl-coenzyme A derived from both acetate and glucose may have exceeded the rate of regeneration of reduced pyridine nucleotide coupled with the breakdown of glucose alone. As a result, a large part of the acetoacetyl-coenzyme A must have escaped reduction and have been deacylated.

Summary. 1. The ketogenic power of various C^{14}-labeled substrates, incubated with actively respiring homogenates of mammary gland and of kidney cortex, has been compared by measuring rate of incorporation of the substrate carbon into carrier-acetoacetate. Whereas acetate and pyruvate were strongly ketogenic, glucose and lactate gave rise to only small amounts of acetoacetate. The analogous behavior of glucose and lactate in contrast with that of pyruvate, indicates that the low ketogenicity of glucose and its ability to stimulate lipogenesis is associated with the regeneration of reduced pyridine nucleotides during its breakdown. 2. No antiketogenic action of glucose was observed when this was added to mammary homogenates metabolizing 1-C^{14} acetate. Under certain conditions, the presence of glucose resulted in the acceleration of the appearance of acetate-carbon in respiratory CO_2 and in acetoacetate. This 'ketogenic' action appeared to be due to stimulation by glucose of the activation of acetate and consequently the formation of acetoacetyl-coenzyme A at a rate exceeding the rate of regeneration of reduced pyridine nucleotide coupled with the breakdown of glucose.

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Effect of Trypsin on Agglutinability of Lipopolysaccharide Treated Erythrocytes.*† (23614)

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Crude somatic antigens of various enteric, Gram-negative bacilli as well as their lipopolysaccharide components become readily attached to erythrocytes of man and sheep, resulting in the acquisition of a new serologic specificity. These modified red blood cells thus become specifically agglutinable in the presence of homologous bacterial antibodies. With modified sheep cells lysis occurs upon the addition of antibody and guinea pig complement. It has been shown that erythrocytes from dog, rabbit, guinea pig, rat, and chicken are as readily modified by *Escherichia coli* antigen as human and sheep erythrocytes(1). Similarly, *Salmonella typhosa* Vi antigen be-

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comes attached to red blood cells from man, sheep, horse, ox, goat, and rabbit(2). Recently, a chance observation revealed that alligator erythrocytes after treatment with *E. coli* antigen were not agglutinated upon the addition of the corresponding antiserum. It was decided, therefore, to explore the possibility that enterobacterial hemagglutination does not occur with erythrocytes from certain cold blooded animals. The present study revealed that red blood cells from alligator and axolotl, following treatment with enterobacterial lipopolysaccharides, are not agglutinated by homologous bacterial antibodies and that trypsin treatment of these red blood cells makes enterobacterial hemagglutination possible.

Material and methods. Crude enterobacterial antigens and highly purified *E. coli* lipopolysaccharides were prepared as described previously(3,4). Blood was procured from man (blood group O), alligator (*Alligator mississipiensis*), axolotl (*Ambystoma mexicanum*), snake (*Elaphe o. obsoleta*), and black bass (*Micropterus sp.*). Trypsin (Difco B454) was dissolved as a 1% solution in phosphate hemagglutination buffer (Difco); this stock solution was kept frozen. Trypsin (0.1%) (2 ml) was freshly prepared and added to the sediment of the erythrocyte suspension (2 ml). The mixtures were incubated in a waterbath at 37°C for 15 minutes. Bacterial antiserums were prepared in rabbits; human serums containing enterobacterial antibodies were obtained from patients infected with the particular microorganisms. The enterobacterial hemagglutination and hemolysis tests were carried out according to the methods described previously(3,4), except for the inclusion of trypsin. Phosphate hemagglutination buffer (Difco) was used as diluent unless indicated otherwise.

Results. Treatment of alligator red blood cells with crude enterobacterial antigens obtained from a variety of enteric microorganisms, including *E. coli, Aerobacter aerogenes, Proteus vulgaris,* and *Pseudomonas aeruginosa,* did not result in agglutination upon the addition of homologous bacterial antiserum obtained from either rabbit or man, nor were positive results obtained with *E. coli* lipopolysaccharides. Neither did hemagglutination occur when sodium chloride solutions (0.85% and 0.6% respectively) were used as diluent instead of buffer, or when the experiments were carried out at 22 or 4°C. In contrast, hemagglutination was always demonstrated in parallel experiments with sheep or human erythrocytes. Failure to obtain agglutination with alligator erythrocytes in the above system is not due to inagglutinability of the red blood cells, since an alligator erythrocyte antiserum prepared in rabbits produced agglutination readily. Limited studies on axolotl, snake, and fish cells also yielded the same results.

Subsequently, it was shown that trypsin treatment of both alligator and axolotl erythrocytes made enterobacterial hemagglutination possible. Aliquots of red cell suspensions from 4 animal species were treated with (1) *E. coli* 111 lipopolysaccharide (5 μg/ml), (2) trypsin (0.1%) followed by *E. coli* lipopolysaccharide (5 μg/ml), (3) *E. coli* lipopolysaccharide (5 μg/ml) followed by trypsin (0.1%), and (4) trypsin (0.1%). The cells were washed 3 times before, between, and after treatments with trypsin and/or lipopolysaccharide. The cell suspensions (0.2 ml) were then added to *E. coli* 111 and *S. sonnei* antiserums in various dilutions (0.2 ml). The mixtures were incubated in a waterbath at 37°C for 60 minutes and centrifuged at approximately 1000 rpm. The resulting hemagglutination was observed grossly and is recorded in Table I.

Perusal of the Table reveals that after treatment with *E. coli* lipopolysaccharide neither alligator nor axolotl cells, in contrast to sheep and human erythrocytes, were agglutinated by the homologous antiserum. However, hemagglutination resulted with both alligator and axolotl cell suspensions when the red blood cells were treated with trypsin either before or after exposure to lipopolysaccharide. It is evident also that trypsin treatment alone did not cause non-specific agglutination by *E. coli* antiserum. Important is the observation that trypsin treatment did not substantially increase agglutination of human or sheep red blood cells, either by producing stronger clumping or agglutination in higher

TABLE I. Effect of Trypsin on Agglutinability of Lipopolysaccharide Treated Erythrocytes.

		Hemagglutination															
		Treatment of erythrocytes															
		LP				Trypsin-LP				LP-trypsin				Trypsin			
Antiserum		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
E. coli 111	1: 100	—	—	4	4	3	3	4	4	3	3	3	4	—	—	—	—
	1: 200	—	—	4	4	3	3	4	4	3	3	3	4	—	—	—	—
	1: 400	—	—	4	3	3	3	4	4	3	3	3	4	—	—	—	—
	1: 800	—	—	3	3	3	3	3	3	3	3	3	3	—	—	—	—
	1: 1600	—	—	2	1	3	2	3	3	1	1	1	1	—	—	—	—
	1: 3200	—	—	±	—	2	1	—	2	1	—	—	—	—	—	—	—
	1: 6400	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1:12800	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S. sonnei	1: 100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1: 1000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

LP = *E. coli* 111 lipopolysaccharide; A = alligator erythrocytes; B = axolotl erythrocytes; C = sheep erythrocytes; D = human (blood group O) erythrocytes; — = no hemagglutination; 1 to 4 = various degrees of hemagglutination.

serum dilutions. Finally, the specificity of the reactions is evident from the fact that a potent *S. sonnei* antiserum did not cause agglutination of any of the erythrocyte suspensions. Additional control antiserums (*E. coli* 55 and 26), not included in the Table, also failed to produce hemagglutination.

Discussion. Enterobacterial hemagglutination takes place in two stages: in the first phase the enterobacterial antigen becomes attached to the surface of the red blood cells without causing visible changes; and in the second phase agglutination ensues upon the addition of homologous bacterial antibodies. The data reported in this communication clearly show that red blood cells from several cold-blooded animal species treated with these antigens are not agglutinated by the corresponding antiserums. This finding is in striking contrast to the results obtained with erythrocytes from sheep and man. The negative findings obtained with alligator and axolotl cells may be due to either failure of lipopolysaccharide attachment or to interference with the agglutination reaction *per se*. It is the second mechanism which accounts for the observed results, since trypsin treatment results in specific hemagglutination after exposure of the red blood cells to the lipopolysaccharide, as documented in Table I. It is conceivable that the receptors for these antigens of alligator and axolotl red blood cells are located more deeply than those of sheep and human erythrocytes, perhaps like valleys surrounded by walls of protein or protein-like material, or that a protein moiety located between 2 polysaccharide molecules interferes with the spacial orientation of the antibody necessary for agglutination. Removal of the protein or part thereof may make agglutination by antibody of antigen-modified cells possible. The former concept appears to be in agreement with ideas expressed by Coombs and associates(5,6) who studied "inagglutinable" bovine red blood cells and demonstrated that agglutination could be effected by building out a chain of anti-globulin and globulin molecules from the globulin of the antibody sensitizing the cells. Regarding the second possibility, attention may be called to the interference with bactericidal action of O antibodies by R antigen-antibody complex located on the same cell(7).

Additional experiments are needed to determine whether erythrocytes from other cold-blooded animals resemble alligator or sheep cells in the enterobacterial hemagglutination test. Also, similar studies should be carried out with the numerous microbial polysaccharide antigens which are operative in this reaction. For a list of these bacterial antigens the reader is referred to a recent review(8). In particular, the minimal number of molecules of various lipopolysaccharides per red blood cell from different animal species necessary for hemagglutination should be determined. It should be emphasized that marked differences exist between various polysaccharide

antigens of bacterial, fungal, and protozoal origin regarding their suitability for red blood cell modification. For instance, one of the antigens prepared by MacPherson and coworkers(9) did not modify red blood cells and the others became effective only after treatment with NaOH. Enhancement of erythrocyte modifying capacity by heat and sodium hydroxide differs markedly in degree with various salmonella lipopolysaccharides(4). Vogel(10) showed that the erythrocyte sensitizing antigen from *Saccharomyces cerevisiae* is more heat labile than that of *C. albicans*. The suggested investigations may yield information on the identity or lack of identity of receptors of red blood cells from various animal species for different antigens and conceivably on the taxonomic positions of the animals.

Boyden(11) reported that ox red blood cells adsorb mallein without being agglutinated by homologous antiserum. It will be interesting to determine whether trypsin treatment of these erythrocytes makes mallein hemagglutination possible. Furthermore, Boyden noted that different amounts of this antigen were required for modification of erythrocytes from various animal species. In agreement with the above hypothesis it is conceivable that with erythrocytes from certain animal species some of the antigen and the corresponding antibody become attached to sites not available for the hemagglutination reaction. The effect of trypsin treatment of these red blood cells on the minimal amounts of antigen required for hemagglutination should be determined.

It has been known for several years that trypsin treatment of erythrocytes, by an as yet incompletely understood mechanism, makes possible the detection of incomplete Rh antibodies(12,13,14,15). Raffel(16) postulated that the Rh antigen is located deeply, that trypsin removes stromal constituents which hinder contact of the fixed antibody with a second cell, and that the combining groups of "incomplete" antibodies are closer together than those of "complete" antibodies. In the system of lipopolysaccharide modified trypsinated red blood cells, reported here, the reaction takes place between a "complete" antibody and an artificially attached antigen. It is not known whether the same mechanism of trypsin action is responsible for both phenomena. A comparative study on the effects of various proteolytic enzymes, including papain and ficin, in the two systems may yield the answer to this question. It may be mentioned that, had experiments been carried out solely with alligator and axolotl cells, hemagglutination conditioned by trypsin erroneously could have been ascribed to the presence of "incomplete" antibodies. This example serves again as an illustration of the difficulties encountered in a precise definition of "incomplete" antibodies.

Summary. A study on hemagglutination of red blood cells from alligator, axolotl, snake, fish, sheep, and man modified by crude enterobacterial antigens and *E. coli* lipopolysaccharide (5 μg/ml) revealed the following results. (1) Hemagglutination of antigen-modified erythrocytes from the above cold-blooded animals does not take place in the presence of homologous antibodies, either at 37, 22 or 4°C. (2) Under identical conditions hemagglutination is readily demonstrated with human and sheep red blood cells. (3) Trypsin treatment of alligator and axolotl cells either before or after modification results in hemagglutination upon addition of homologous bacterial antibodies. (4) It is concluded that trypsin removes an inhibitor interfering with the agglutination reaction *per se* and not with the attachment of the antigens to the red blood cells of alligator and axolotl.

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Effects of Air Ions on Isolated Rabbit Trachea.* (23615)

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Evidence has recently accumulated indicating that negative air ions are beneficial in certain cases of hay fever and asthma(2). Positive ions, on the other hand, are reported to produce nasal obstruction, dryness of the mucous membranes, and headaches(6,8). In order to test whether these clinical observations could be correlated with any measurable changes in the pulmonary clearing mechanism (including ciliary rate, rate of mucus flow, and smooth muscle tone), the following *in vitro* experiments were undertaken.

Methods. Pieces of fresh rabbit† trachea 2-3 cm long were cut along the anterior wall, spread open, and pinned to small blocks of wood. These blocks were then placed in glass-and-plastic chambers (Fig. 1) containing air of 80-100% relative humidity. By means of a 10X dissecting microscope and a Strobotac-Strobolux combination it was possible to observe the surface of the tissue and determine the ciliary rate ± 50 beats/min. Rate of mucus flow was determined by timing the progress of air-bubbles or of added grains of talc (averaging .01 mm in diameter) in the mucous layer. Clearing efficiency was gaged by the ability of a tissue to remove talc grains applied evenly over its surface with a medical powder blower. The experiments were conducted at room temperature, which varied from 21-23°C. Air ions were generated by beta radiation derived from tritium which was contained in sealed foils; a reversible rectifying circuit made it possible to select positive or negative air ions at will(5). Measurements made with the Beckett probe and Beckman micro-microammeter(3) established that 1 x 10^9 air ions of either charge impinged on each cm^2 of exposed tissue/sec.

Results. 1. *Effect on Ciliary Rate.* Under conditions described above, the initial ciliary rate averaged between 1400 and 1500 beats/min. This level was maintained for a few hours, then slowly declined. In most experiments ciliary activity ceased within 6 hours, although in a few cases it continued for 24 hours. The manner in which rabbits were

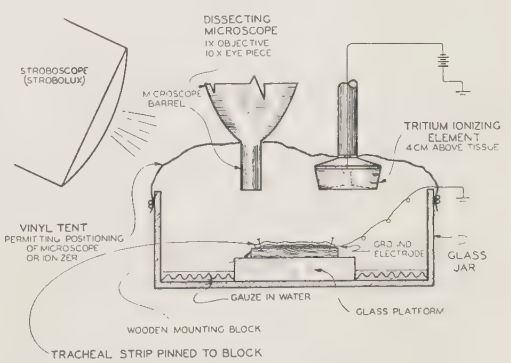

FIG. 1. Arrangement for observing effect of air ions on isolated rabbit trachea. Three such units were available.

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† We express our warm appreciation to Drs. Go Ing Gan and Agus Suhada of the University of Indonesia for meticulous work in a series of survey experiments with frogs, rats, guinea pigs, and cows in this laboratory during the spring of 1957. Their results demonstrated clearly that tracheas of these animals are not suited to *in vitro* technics we have employed and led to our selection of the rabbit as the experimental animal.

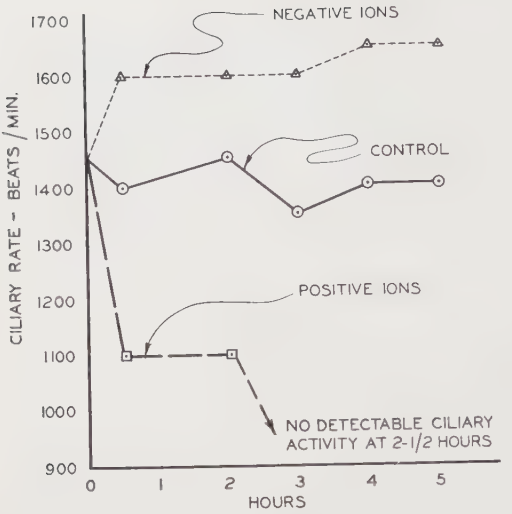

FIG. 2. Effect of air ions on ciliary activity of isolated rabbit trachea.

sacrificed had a considerable effect on vigor and durability of ciliary activity; intraperitoneal injection of a lethal dose of nembutal was found most satisfactory. Exposure of the tracheal strip to positive ions caused the ciliary rate to drop to 1100/min., and in some instances to cease altogether. Negative ions produced a rise in ciliary rate to 1600/min., or occasionally as high as 1700/min. (Fig. 2). If the ciliary rate of a tissue was lowered to 1100/min. by short exposure to positive ions, and the tissue then placed in a control chamber, the cilia would continue to beat at this low rate until death of the tissue. If treatment with negative ions was instituted during this period of lowered ciliary activity, the rate was promptly restored to normal or above (Fig. 3).

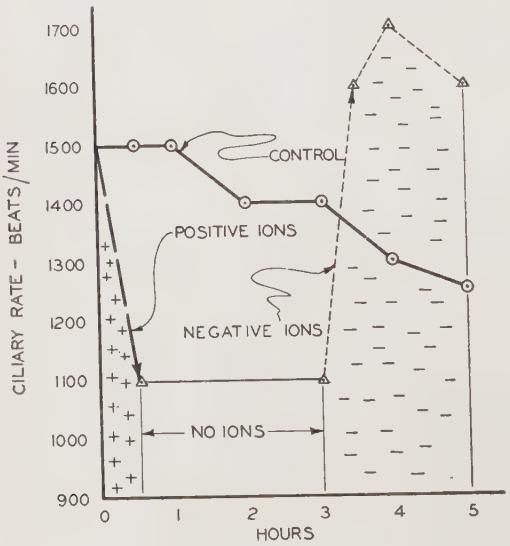

FIG. 3. Capacity of negative air ions to reverse inhibition of ciliary activity induced by positive air ions acting on isolated rabbit trachea.

2. *Effect on Mucus Flow.* As shown by Dalhamn and others(1), the ciliary rate and rate of mucus flow do not necessarily parallel each other. However, in most of our experiments exposure to positive ions caused the mucus flow and clearing ability either to decrease markedly or stop altogether. Negative ions, on the other hand, increased the mucus flow rate in about half of our experiments, had no effect on the mucus flow rate in the other half. (Fig. 4).

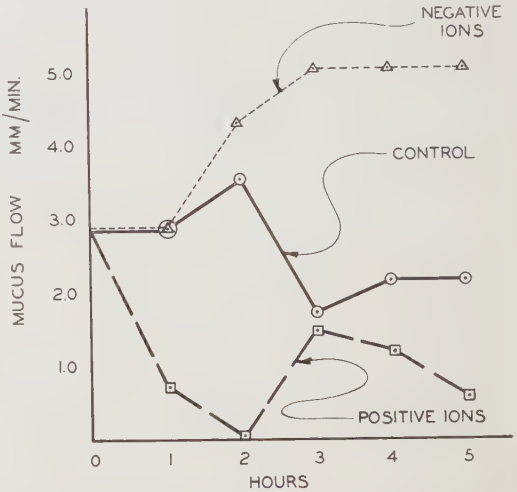

FIG. 4. Effect of air ions on rate of mucus flow on surface of isolated rabbit trachea. Rate measured by following movement of talc particles or air bubbles.

3. *Effect on Smooth Muscle.* It was routinely observed that positive ions caused the membranous posterior wall of the trachea to contract; in this condition it was impossible to elicit peristalsis by stretching the tissue laterally. Negative ions completely reversed this effect; the posterior wall relaxed and peristalsis could once more be elicited.

4. *Effect on the Mucous Layer.* Upon exposure to positive ions, the tracheal surface generally assumed a characteristic dry, nonglossy appearance. Negative ions either had

no effect on the surface or caused the appearance of a watery fluid, which could be drained off. Whether this fluid was a condensation of water molecules from the air or represented an actual secretion is not known. The mucous layer of control tissue had a glossy appearance, but was never watery.

5. *Effect on Ciliary Resistance to Mechanical Trauma.* It was noted that positive ions rendered the cilia peculiarly vulnerable to mechanical trauma. A single, very gentle stroke with a cotton-tipped swab dipped in Ringer's solution, which had no lasting effect on the control, completely stopped ciliary activity over all or most of the surface of the tissue receiving positive ions. This enhanced vulnerability completely disappeared when the tissue was exposed to negative ions. It also disappeared if the tissue was allowed to stand in the control chamber for an hour or more.

It is not at all obvious on *a priori* grounds that air ions should possess any capacity to influence biological systems. Nevertheless, evidence is collecting which establishes such activity in several areas. For example, positive ions reduce the succinoxidase content of the rat adrenal gland(4), and negative ions raise the CO_2 combining power of hamster blood plasma(7). During the past 2 years we studied the response of microorganisms suspended in very small drops of water when exposed to positive and negative air ions and have secured quantitatively reproducible alterations in survival curves(3).

While we have as yet developed no theory to account for the action of air ions on the rabbit trachea, it is possible that some of the changes we noted in the rate of water evaporation from droplets containing bacteria(3) may have significance here as well. A study of the inter-relationships and possible interdependence of the effects described in this series of experiments is currently in progress.

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Effect of Sodium Lactate Infusion on Urate Clearance in Man.* (23616)

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Gibson and Doisy(1) made the interesting observation, in normal human subjects, that ingestion of sodium lactate results in a striking if transitory decrease in the urinary excretion of uric acid, accompanied by a slight rise in plasma uric acid, suggesting "an elevated threshold in the kidney." Quick(2), Michael (3) and Nichols *et al.*(4) confirmed the decline in urinary uric acid excretion, a phenomenon which, in view of the physiological significance of lactic acid as a metabolic intermediate, may ultimately throw some light upon the still obscure nature of the renal tubular transport mechanisms for uric acid. The present study was designed to define this effect of lactate on renal excretion of uric acid more precisely by use of standard clearance technics.

Methods. Gouty subjects, in the intercriti-

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cal phase of the disorder, were employed in order to provide a wider range of urinary uric acid excretion levels. The 13 subjects studied were maintained on a low purine diet prior to study. Clearance measurements were made in the morning, with the patients in the post-absorptive state. Liberal fluid intake ensured adequate urine flow. Standard technics were employed(5). In all subjects glomerular filtration rate was measured by the inulin clearance, effective renal plasma flow by the para-aminohippurate clearance. Every experiment included three 15-minute control periods for determination of C_{inulin}, C_{PAH} and C_{urate}. The effect of lactate infusion on the maximal tubular excretory capacity was studied in one case by Tm_{PAH} estimations. The urine and blood pH was measured in a Cambridge pH meter immediately after procurement of the samples. Urate, inulin and PAH were determined by methods previously indicated(6).

Results. Effects of infusion of sodium r-lactate. Five experiments were performed; 2 with .17 M lactate, 2 with .5 M, 1 with .4 M. Representative results with each dosage are cited in Table I. C_{inulin} and C_{PAH} were not significantly altered(7). Tm_{PAH}, in the 1 case examined, transiently increased(7,8). A striking decline in urate excretion was consistently observed. This was uniformly apparent in the first 15-minute urine collection period and, when sufficient quantities of lactate were injected, became progressively more pronounced over the approximately 2-hour period of sustained infusion. In no instance, however, did C_{urate} decline to less than 1.0 ml/min. In the 5 experiments, the mean control UV_{urate} of 972 μg/min. fell to a mean minimum of 125 μg/min.; C_{urate} from 10.4 ml/min. to 1.4 ml/min.; and C_{urate}/C_{inulin} from 8.1% to 1.1%. An unequivocal rise in serum urate levels during the period of infusion was not demonstrated, presumably because of the brief duration of study. The urine pH invariably became alkaline, without any demonstrable change in blood pH.

Effect of sodium r-lactate on salicylate uricosuria. The uricosuria produced by injection of salicylate in appropriate dosage was found to be abolished, at least temporarily, by concomitant infusion of sodium lactate in adequate concentration. Thus in Case 4 (Table I) the mean control C_{urate}, increased from 11.8 ml/min. to a mean of 51.2 ml/min. during salicylate infusion, temporarily fell to 17.4 ml/min. after addition of .4 M sodium lactate to the continued salicylate infusion. Thereafter, C_{urate} gradually returned to the high levels observed with injection of salicylate alone, despite continued lactate infusion. Parallel changes occurred in C_{urate}/C_{inulin}. (It should be noted that the apparent wearing off of the suppressant effect of lactate on salicylate uricosuria may be due, in part, to enhancement of the uricosuric effect of salicylate when the urine is made alkaline by administration of sodium bicarbonate(9) or its equivalent.) Two analogous experiments in which .17 M instead of .4 M sodium lactate was employed failed to demonstrate any inhibition of salicylate uricosuria.

Effect of sodium r-lactate on probenecid uricosuria. In a parallel experiment with probenecid (Table I), the mean control C_{urate} of 9.1 ml/min. was increased to a maximum of 41.3 ml/min. by intravenous injection of .5 gm probenecid; upon addition to the probenecid infusion of sodium lactate in .4 M concentration, C_{urate} steadily decreased to a minimum of 4.6 ml/min., a decline which was sustained. C_{urate}/C_{inulin} rose and fell concomitantly. The effect of probenecid on the retention of urate caused by lactate was studied in two additional subjects by reversing the order of drug administration. The results shown in Fig. 1 are illustrative. The mean C_{urate}/C_{inulin} in the pre-medication period was 3.8%. After infusion of .17 M sodium lactate for 40 minutes at a rate of 14 ml/min., C_{urate}/C_{inulin} fell to 0.9%. At this point 1.5 g probenecid was given by mouth, the lactate infusion being continued for one hour. After 20 minutes C_{urate}/C_{inulin} began to rise, more rapidly when the lactate infusion was discontinued. The course of the $C_{urate}/C_{creatinine}$ thereafter reflected the usual probenecid uricosuria.

Effect of sodium r-lactate on urate retention caused by pyrazinamide. C_{urate} was reduced by administration of 1.0 g pyrazinamide(10) from the premedication 7.4 ml/min. to 4.3 ml/min. (Table I). Upon infusion of .4 M

sodium lactate, C_{urate} declined further to a minimum of 1.3 ml/min., in part doubtless due to the continued action of pyrazinamide. Since the final level of C_{urate} was not lower than ordinarily obtained with pyrazinamide or lactate alone, an additive or potentiating ef-

TABLE I. Effect of Sodium Lactate Infusion on Urate Clearance, Salicylate and Probenecid Uricosuria, and Urate Retention Caused by Pyrazinamide. (All infusions given intravenously at 8 ml/min.)

Age, B.S.A.	Period (min.)	C_{inulin} (ml/min.)	C_{PAH} (ml/min.)	UV_{urate} (μg/min.)	C_{urate} (ml/min.)	Urine pH
39, 2.04 M^2	−58 to 0	106	410	672	7.4	5.4–5.6
	.17 M sodium lactate, 1000 ml					
	0– 25	92.7	367	300	3.3	6.0
	25– 45	104	415	160	1.8	6.8
	45– 86	106	413	170	1.9	7.3
	86–129	102	425	182	1.9	7.5
39, 2.27 M^2	−45 to 0	145	681	935	9.5	5.5–5.7
	.4 M sodium lactate, 1000 ml					
	0– 15	176	860	675	6.9	6.1
	15– 30	143	715	141	1.4	6.6
	30– 60	130	612	115	1.2	7.1
	60– 90	136	637	115	1.2	7.6
	90–120	153	700	120	1.2	7.7
42, 2.07 M^2	−115 to −85	124	459	688	7.1	6.8
	− 85 to 0	117	80.7*	838	9.0	5.9
	.5 M sodium lactate, 1000 ml					
	0– 22	120	82.6*	415	4.2	5.9
	22– 40	118	92.6*	148	1.5	6.6
	40– 80	120	81.2*	145	1.5	7.0
	80–121	120	—	113	1.1	7.5
39, 2.20 M^2	−30 to 0	124	669	1085	11.8	—
	Sodium salicylate, 3.0 g, in 100 ml saline solution					
	0– 31	122	830	3302	41.3	6.5
	31– 42	145	770	3790	56.5	—
	42– 55	145	790	3727	55.7	6.9
	Sodium salicylate, 4 g, in .4 M sodium lactate, 1000 ml					
	55– 75	110	895	1165	17.4	6.8
	75– 95	133	917	2038	30.4	7.1
	95–136	108	872	3523	52.6	7.3
	136–165	110	830	3450	51.5	7.6
60, 1.58 M^2	−30 to 0	89.3	311	919	9.1	6.2–6.4
	Probenecid, .5 g, in 100 ml saline solution					
	0– 15	71.7	260	2050	20.7	6.4
	15– 30	93.9	273	3230	35.9	6.6
	30– 45	86.8	280	3225	41.3	6.7
	Probenecid, .5 g, in .4 M sodium lactate, 1000 ml					
	45– 65	95.1	319	1828	22.0	6.9
	65– 87	93.2	291	653	7.5	7.3
	87–125	71.9	244	417	4.6	7.6
	125–165	71.0	296	448	4.7	7.6
37, 2.05 M^2	−28 to 0	127	469	571	7.4	5.6
	1.0 g pyrazinamide orally					
	0– 22	117	483	530	6.9	5.7
	22– 41	120	503	482	6.3	5.9
	41– 61	130	475	330	4.3	5.8
	.4 M sodium lactate, 1000 ml					
	61– 86	132	505	197	2.6	6.2
	86–105	123	577	100	1.3	6.9
	105–125	123	571	100	1.3	7.5
	125–135	116	586	113	1.5	7.6

* Tm_{PAH} (mg/min.).

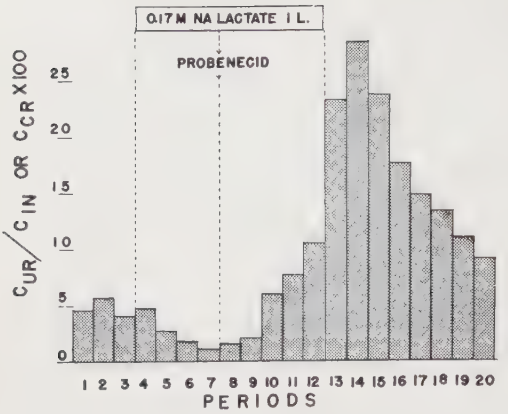

FIG. 1. Gouty subject. The first 3 clearance periods, each of 10 min duration, indicate C_{urate}/C_{inulin} before medication. In periods 4 through 12, a total of 1 liter of 0.17 M sodium lactate was infused at approximately 10 ml/min. At point indicated by arrow, 1.5 g probenecid was given by mouth. Initial reduction in C_{urate}/C_{inulin} produced by infusion of 0.17 M sodium lactate was gradually overcome by uricosuric action of the probenecid. Upon cessation of lactate infusion, however, the probenecid effect was much more pronounced (post-infusion periods each of 1-2 hours duration, endogenous creatinine clearance used as measure of filtration rate).

fect is not suggested.

In another experiment the initial C_{urate} of 6.8 ml/min. was first reduced by infusion of .4 M sodium lactate to 1.7 ml/min.; then, with continued lactate infusion, pyrazinamide, 1.0 g was given orally. The minimum C_{urate} resulting was 1.3 ml/min.

Discussion. The striking reduction in urinary uric acid excretion effected by lactate is of particular interest because no other natural metabolite has yet been demonstrated to share this property in such pronounced degree. High fat diets cause similar but apparently less marked uric acid retention(11-13), presumably through the action of some metabolic intermediate (acetoacetic acid?(2)). Pyruvate causes an increase in urinary uric acid excretion(1,2); but even though administration of lactate may result in a distinct rise in blood pyruvate (and α-ketoglutarate) levels(14), the predominant effect on uric acid excretion is that of lactate. Administration of citrate (as the potassium salt, 25 g by mouth over a period of 50 minutes) caused an increase in C_{urate}/C_{inulin} from 4.8 to 8.8% in one hour(15). Intravenous infusion of .4 M acetate likewise increased the urate clearance to about 2-fold; lesser concentrations of acetate had no measurable effect(15).

Alkalinization with .4 M sodium bicarbonate by sustained intravenous injection caused an equivocal rise in C_{urate}/C_{inulin}, from a mean of 8.0% to a mean of 9.7%(9). Alkalinization of the urine by means of Diamox slightly decreased C_{urate}/C_{inulin}, from a mean of 6.4% to a mean of 5.3%(9), in most cases associated with an approximately 10% reduction in glomerular filtration rate.

The possible physiological role of lactic acid in the regulation of renal excretion of uric acid has long been a subject of interest, notably in connection with the lacticacidemia and reduced urinary uric acid excretion of exercise (16). Nichols *et al.*(4), using clearance technics, found that the sharp decrease in urate clearance observed *during* exercise might be accounted for by the accompanying reduction in glomerular filtration rate, but persisted long after the filtration rate had returned to normal upon cessation of exercise; the increased plasma lactic acid levels also persisted. Moreover, there appears to be a significant relationship between the height of the plasma lactic acid level and the degree of uric acid retention(3,4). These observations would suggest that the metabolism of lactic acid may be a factor in the regulation of uric acid excretion, at least under the conditions of exercise.

The present study makes clear that infusion of sodium lactate causes no reduction in the filtered urate loads presented to the tubules and implies that excessive lactate in the glomerular filtrate and/or blood enhances tubular reabsorption or, possibly, decreases tubular excretion of urate. It is known that filtered lactate is completely reabsorbed by the tubules, at least until the Tm for lactate is approached, at blood lactate levels of 60-100 mg %(17,18); such high blood levels are attained with strenuous exercise but not by injection of lactate in the concentrations here employed.

Summary. Renal clearance studies are described which corroborate and extend earlier observations indicating that administration of sodium lactate in sufficient dosage results in

a profound fall in urinary excretion of uric acid. C_{urate} declined from a mean control level of 10.4 ml/min. to a mean minimum of 1.4 ml/min. The uricosuria produced by salicylate and probenecid was temporarily abolished by lactate. The significance of these findings is discussed.

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Quantitative Measurement of Inhibition of the Enzymatic Detoxification of Malathion by EPN (ethyl p-nitrophenyl thionobenzenephosphonate).* (23617)

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Frawley *et al.*(1) recently demonstrated that simultaneous administration of ethyl p - nitrophenyl thionobenzenephosphonate (EPN) and S-(1,2-dicarbethoxyethyl)-O, O-dimethyl phosphorodithioate (malathion) to rats and dogs causes marked potentiation of the acute and subacute toxicity of these insecticides. The widespread use of organic phosphorus-containing insecticides on food crops makes it possible for the diet of man and domestic animals to contain low quantities of several of these compounds. For an accurate evaluation of the health hazards which might result from potentiation of the action of cholinergic organic phosphates it was desirable to have a sensitive, direct method for measuring the biochemical event responsible for the effect which could be applied to the tissues of animals fed low levels of these insecticides. It has been observed by Cook *et al.*(2) that EPN inhibits the hydrolytic detoxification of malathion by rat liver homogenates *in vitro*. This observation provides a possible explanation for potentiation of the toxicity of malathion by EPN since the low mammalian toxicity of malathion is apparently highly dependent upon rapid and complete detoxification. The present communication describes a quantitative method for measuring inhibitory action of EPN on detoxification of malathion. Data are presented which show the applicability of this method for *in vitro* experiments and for measurements on tissues taken from animals given acutely toxic and low dietary levels of EPN. The tissue distribution of the esterase which catalyzes the hydrolytic detoxification of malathion was also studied.

Methods and materials. Adult male and

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female Sprague-Dawley rats (250-400 g), male and female Carworth Farms mice (25-35 g) and adult male guinea pigs (*ca* 300 g) were used for this study. The rats and mice were fed Rockland Rat Diet and the guinea pigs received Rockland Rabbit Pellets. For experiments on the metabolism of malathion the animals were anesthetized with ether and blood was withdrawn by cardiac puncture. The animals were then sacrificed and the tissues were quickly removed, weighed and homogenized in cold water. Solutions of malathion or EPN (0.1 M) were prepared for the enzyme studies by dissolving the compounds in ethanol and diluting to the desired concentration with distilled water. For *in vivo* experiments solutions of EPN dissolved in 10% ethanol and 90% propylene glycol and undiluted malathion were given intraperitoneally. Chemical conversion of malathion to its oxygen analogue (malaoxon) was accomplished by a modification of the method of Fallscheer and Cook(3). Thus 2 ml of water and 3 ml of dilute bromine water (0.1 ml of saturated bromine water in 25 ml of distilled water) were added to 5 ml of 1×10^{-3} M technical malathion (95%). This solution was allowed to stand for one hour during which time complete oxidation of malathion occurred. The concentration of bromine present in the oxidized malathion solution did not interfere with the activity of the enzyme systems to which it was added. The bromine-treated malathion produced 50% inhibition of rat brain cholinesterase at a final molar concentration of 1.6×10^{-6}. Enzymatic conversion of malathion to malaoxon was accomplished by the method of Murphy and DuBois(4) using 1×10^{-4} M malathion as the substrate. Cholinesterase measurements were performed manometrically by the method of DuBois and Mangun(5). The amount of oxidized malathion produced or destroyed during incubation with tissue homogenates was measured by a bioassay procedure using the cholinesterase system in the manner which we described previously(4).

Results. To perform quantitative measurements of the inhibitory effect of EPN on the detoxification of malathion a method was needed for measuring the activity of the enzyme which catalyzes the hydrolytic detoxification of malathion and its toxic oxygen analogue (malaoxon) in animal tissues. A number of preliminary experiments on the optimum conditions for measuring the activity of the esterase resulted in the development of a procedure which satisfied the requirements of a quantitative enzyme assay. The oxygen analogue of malathion was employed as the substrate in the test system since it has strong anticholinesterase activity in contrast to pure malathion which has no action on this enzyme *in vitro*. It was possible to utilize the anticholinesterase activity of the oxygen analogue (malaoxon) to bioassay the quantity of malaoxon destroyed by the esterases of animal tissues. The test system contained 0.15 ml of 5×10^{-4} M malaoxon, 0.4 ml of 0.1 M phosphate buffer (pH 7.2), 0.1 or 0.2 ml of cold, aqueous tissue homogenate and enough distilled water to make a final volume of 3 ml. Assays were always performed in duplicate using 2 levels of tissue. Thus 0.1 ml and 0.2 ml of 2.5% liver (2.5 mg and 5 mg) and 10 and 20 mg of serum were used except in those experiments in which the inhibitory effect of EPN necessitated the use of higher quantities. The tubes containing all of the constituents of the reaction mixture except the substrate were placed in a constant temperature bath at 38°C and the malaoxon was then added. After incubation for 10 minutes aliquots (0.6 ml) were withdrawn and added immediately to the cholinesterase test system(5) containing 50 mg of homogenized rat brain as the source of the enzyme. From the amount of inhibition of cholinesterase produced by malaoxon before and after incubation with tissue homogenates it was possible to calculate the quantity of malaoxon which was detoxified by using the logarithmic relationship between inhibition and malaoxon concentration shown in Fig. 1.

In the test system described above the rate of detoxification of malathion was dependent upon the tissue concentration. Thus 2.5 and 5 mg of liver detoxified 9.1 and 20.1 μg of malaoxon respectively. Under the conditions selected for the assay the reaction rate was linear with time as evidenced by detoxification of 4.7, 8.9 and 13.7 μg of malaoxon by 2.5 mg

TABLE I. Detoxification of Malaoxon by Tissues of Various Species.

Tissue	μg of malaoxon destroyed/mg tissue/10 min.			
	Rats	Mice	Guinea pigs	Dogs
Liver	4.03 ± .17*	1.84 ± .15	3.74 ± .28	11.5 ± 1.1
Kidney	.66 ± .04	.46 ± .05	.21 ± .13	1.85 ± .66
Serum	.99 ± .07	1.78 ± .08	.19 ± .03	.08 ± .06
Lung	.75 ± .05	.58 ± .02	.19 ± .13	1.18 ± .11
Ileum	.39 ± .02	.23 ± .03	.13 ± .04	.07 ± .02
Spleen	.12 ± .03	.03 ± .01	.16 ± .12	.03 ± .03

* Figures represent avg values ± avg dev. from mean.

of liver in 5, 10 and 15 minutes respectively. Liver homogenates heated at 100°C for 2 minutes lost their ability to catalyze the detoxification of malaoxon and dialysis against distilled water for 18 hours at 5°C caused only 6% loss of enzyme activity. Storage of whole liver homogenates at 5°C for 18 hours caused only 10% loss of activity.

The first application of the method for measuring the detoxification of malaoxon consisted of a survey of the enzyme activity of tissues from various species. The results of these measurements are summarized in Table I in which each value is an average of assays on the tissues of at least 3 animals. These measurements demonstrated that liver exhibits the highest activity in all species used with dog liver being the most active. The serum of mice and rats was capable of detoxifying a considerable quantity of malaoxon but dog serum exhibited no activity. Kidney and lung also contained the detoxifying enzyme. Other assays conducted on female mice and rats indicated that there is no sex difference in the enzyme activity of the liver of mice and the serum of mice and rats. However, the activity of the liver of male rats was about 4 times as great as that of females as evidenced by the detoxification of 4 (range 3.4 to 4.5) μg of malaoxon/mg of male rat liver and 0.99 (range 0.9 to 1.1) μg/mg by the livers of female rats.

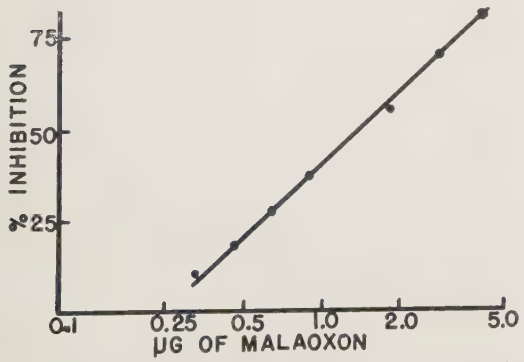

FIG. 1. Inhibitory effect of malaoxon on brain cholinesterase *in vitro*. Ordinate, % inhibition; abscissa, concentration of malaoxon plotted logarithmically.

Addition of EPN to the enzyme system which catalyzes the detoxification of malaoxon markedly inhibited the reaction. When various concentrations of EPN were incubated with homogenized liver for 5 minutes prior to addition of malaoxon the amount of inhibition of the detoxifying esterase was proportional to the logarithm of the EPN concentration within the range of EPN concentrations producing between 20% and 80% inhibition. In these tests 50% inhibition of the enzyme activity of male rat liver occurred with a final concentration of 2.8×10^{-5} M EPN and the lower activity of the liver of female rats was inhibited to the same extent by 5×10^{-6} M EPN. To ascertain whether this inhibitory effect also occurs *in vivo* a group of 3 adult male rats was given one-half of the LD_{50} of EPN (13 mg/kg) intraperitoneally and one hour later the animals were sacrificed for measurements of the enzyme activity of serum and liver. Complete inhibition of the ability of the serum and liver to detoxify malaoxon was observed. This finding was followed by similar experiments in which several lower doses of EPN were tested to find the quantity which produced partial inhibition of the detoxification enzyme. The results summarized in Fig. 2 represent the average of assays performed on tissues from groups each containing at least 3 male rats which received 0.5, 1 or 1.5 mg/kg of EPN intraperitoneally. The per cent inhibition of enzyme activity was calculated from the assay results using the normal values shown in Table I as a basis for the

calculations. The data in Fig. 2 show that 84% and 93% inhibition of the ability of liver and serum of male rats to detoxify malaoxon occurred in 1 hour after the administration of 1.5 mg/kg of EPN. Significant inhibition was also observed with 0.5 mg/kg of EPN which is approximately one-fiftieth of the LD_{50}. The effect of inhibition of the detoxifying esterase by EPN on the susceptibility of rats to the acute toxicity of malathion was demonstrated by determining the LD_{50} of malathion to male rats treated 1 hour previously with 1.5 mg/kg of EPN. The approximate LD_{50} of malathion to these animals was 550 mg/kg as compared with 1100 mg/kg for comparable controls. The dose of EPN used in this experiment did not cause any inhibition of the cholinesterase activity of brain,

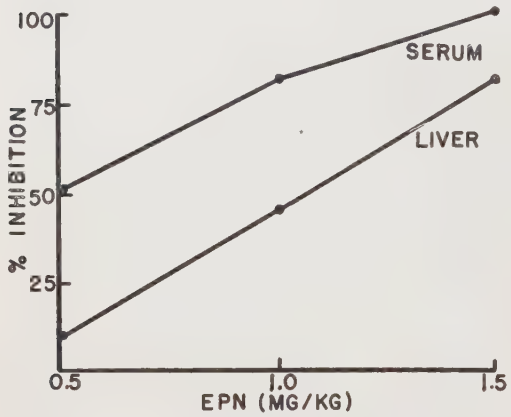

FIG. 2. Inhibitory effect of EPN *in vivo* on ability of rat tissues to detoxify malaoxon. Assays performed on tissues taken from rats 1 hour after intraperitoneal administration of EPN.

serum or submaxillary glands and the increased toxicity of malathion can, therefore, be attributed to inhibition of its normal detoxification.

The duration of the inhibitory effect of EPN was studied by administering 1.5 mg/kg of EPN to a series of male rats and sacrificing them at various intervals for assays on serum and liver. The results of these measurements are shown in Fig. 3 in which each value on the curves represents an average for the tissues of 3 animals. Slow reversal of the enzyme inhibition was observed as evidenced by a gradual return of the activity during a 72-hour observation period.

TABLE II. Effect of Feeding EPN on the Ability of Rat Tissues to Detoxify Malaoxon.

Dietary level of EPN (ppm)	μg of malaoxon destroyed/mg tissue/10 min.		% inhibition	
	Liver	Serum	Liver	Serum
Control	5.6 ± .72	1.52 ± .23		
5	4.0 ± .15	.92 ± .10	28.6	39.5
10	2.4 ± .5	.62 ± .18	57.1	59.2
20	1.2 ± .15	.29 ± .12	78.6	80.9
50	.62 ± .09	.13 ± .02	87.9	91.4
100	.28 ± .13	.07 ± .03	95.0	95.4

It was considered important to ascertain the effects of diets containing EPN on the activity of the enzyme which detoxifies malaoxon since EPN is used as an insecticide on food crops. For this experiment groups each containing 4 male rats were fed various levels of EPN in the diet for 2 weeks and were then sacrificed for measurement of the ability of liver and serum to detoxify malaoxon. The results of these assays are summarized in Table II where it may be seen that levels as low as 5 ppm of EPN caused inhibition of the detoxification enzyme. The amount of inhibition was dependent upon the dietary level of EPN over the range of 5 to 100 ppm of EPN with nearly complete inhibition being observed at the highest level.

The inhibitory action of EPN on the detoxification of certain cholinergic organic phosphates has a useful application in research on the enzymatic conversion of thiophosphates to their corresponding oxygen ana-

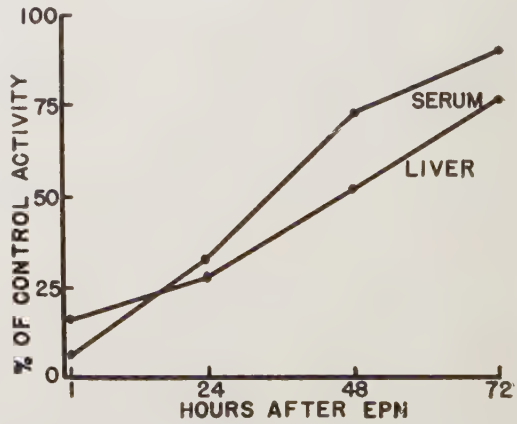

FIG. 3. Duration of inhibitory effect of a single intraperitoneal dose of EPN (1.5 mg/kg) on ability of rat tissues to detoxify malaoxon.

logues. In a previous communication(4) we described experiments which demonstrated that the enzymatic conversion of thiophosphates to their oxygen analogues *in vitro* is complicated by coincident hydrolytic destruction of the compounds and their active metabolites. The strong inhibitory action of EPN on the detoxification of malathion suggested the use of EPN to inhibit destruction during studies on the enzymatic conversion of malathion to malaoxon by liver homogenates. When malathion (1×10^{-4} M) was incubated with 2.5 to 25 mg of whole liver homogenate using the test system described by Murphy and DuBois(4) no accumulation of malaoxon occurred in the reaction mixture. However, homogenates prepared from the livers of 5 male rats which were sacrificed at 1 hour after intraperitoneal administration of 13 mg/kg of EPN produced an average of 1 μg of malaoxon/mg of liver/10 minutes. The results of this experiment demonstrated that the conversion of malathion to its cholinergic oxygen analogue can be studied *in vitro* using whole liver homogenates by inhibiting the enzyme responsible for the breakdown of the parent compound and its active metabolite with EPN. It seems likely that this same procedure can be employed for studying the metabolic conversion of some of the other insecticidal thiophosphates to toxic metabolites.

Discussion. The present investigation resulted in the development of a quantitative method for measuring the enzymatic detoxification of malathion and its active metabolite, malaoxon, by mammalian tissues. A survey of tissues from various species revealed that liver, serum, kidney and lung contained the highest concentrations of the enzyme with the exceptions being very low activity in the serum of guinea pigs and dogs and the lungs of guinea pigs. In the present study no attempt was made to identify the degradation products of malathion or malaoxon but other investigators(2,6,7) have presented evidence that the detoxification consists of hydrolysis of the ester linkages in the 1,2-dicarbethoxyethyl side-chains. It seems reasonable to assume that the esterase which catalyzes this reaction may function in the detoxification of various ester-type drugs and food constituents.

The marked inhibitory action of EPN on the detoxification of malaoxon *in vitro* is in agreement with the findings of Cook *et al.*(2) and provides an explanation for the ability of EPN to potentiate the toxicity of malathion. More direct evidence in support of this possibility was obtained in the present study by the parenteral administration and feeding of EPN to rats. Single doses of EPN, below those which affect cholinesterase activity, markedly inhibited the ability of serum and liver to detoxify malaoxon and greatly increased the toxicity of injected malathion. The feeding of diets containing quantities as low as 5 ppm of EPN for 2 weeks caused significant inhibition of the detoxification enzyme. This enzyme system is thus considerably more sensitive toward inhibition by EPN than is cholinesterase because the lowest dietary level of EPN which produces significant inhibition of cholinesterase in a period of 2 weeks has been found(8) to be 25 ppm. On the basis of our finding that reversal of inhibition of the detoxifying esterase is relatively slow EPN may exert a cumulative toxic effect on this enzyme when fed in the diet for prolonged periods of time. Since inhibition of the enzyme was demonstrated by feeding levels of EPN near the maximum amount (3 ppm) which is currently permitted as a residue in food crops further studies are indicated to determine the influence of dietary EPN on the detoxification of various organic phosphates and drugs containing ester linkages.

Summary. A quantitative method for measuring the hydrolytic detoxification of malathion and its toxic metabolite, malaoxon, was developed. Application of the method to normal tissues revealed that the liver of several species contains the highest concentration of the enzyme but serum, kidney and lung also exhibited activity in some species. EPN was found to inhibit the enzyme system which detoxifies malathion *in vitro* and *in vivo*. This interference with the detoxification of malathion by EPN provides an explanation for the potentiation of toxicity which occurs when the two compounds are administered simultaneously and suggests the possibility that EPN may inhibit the detoxification of other esters which utilize the same detoxification

pathway.

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Isolation of Nutritional Variants from Conjunctival and HeLa Cells.* (23618)

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Properties acquired by human cells upon prolonged cultivation have been a subject of considerable interest. Attention, however, has been directed chiefly toward the problem of acquired malignancy as evidenced by the numerous publications on this subject(1). While changes in the nutritional requirements of various laboratory cell strains have long been suspected, a report on the appearance of a variant capable of utilizing a chemical compound in lieu of an essential nutrient to support growth has been conspicuously lacking. Those of Haff and Swim(2), Puck(3) and Chang(4) referred to quantitative differences in the requirement of, or tolerance to, certain biologicals of unknown complex composition. This publication describes the successful isolation of nutritional variants from our cultures of human conjunctival(5) and HeLa cells(6). These variants are capable of utilizing certain chemical compounds in lieu of glucose to support continuous cell growth.

Material and methods. The maintenance of stock cell cultures; collection, storage and dialysis of serum; composition of the basal carbohydrate-free medium; glucose oxidase test; enumeration of cell number, and, assessment of cell multiplication rate have already been described(7). *Compounds.*† D(+)xylose, d-ribose, d-arabinose, sodium lactate, sodium pyruvate and alanine were used. These compounds were tested for possible trace contamination with glucose by the glucose oxidase test, and for their ability to prevent progressive degeneration of the stock conjunctival cells at concentrations of 125, 25 and 5 mM in the basal media. With the exception of d(+)xylose, these compounds gave negative glucose oxidase tests at concentrations of 2M, and were unable to prevent progressive cell degeneration. The three preparations of d(+)xylose tested gave positive glucose oxidase reactions at concentration of 0.1 to 0.2 M, and 2 of them were able to prevent progressive cell degeneration at concentrations of 25 and 125 mM. Although treatment of these xylose preparations with glucose oxidase, as described(7), failed to reduce appreciably their reactivities in the glucose test,‡ their ability to prevent cell degeneration was abolished. Thus, d(+)xylose which has been pretreated with glucose oxidase was used exclusively in this study. *Isolation of variants.* About one million cells

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† D(+)xylose, C. P., was purchased from Pfanstiehl, Fisher and Nutritional Biochemicals; d-ribose, d-arabinose and sodium pyruvate, from Nutritional Biochemicals; sodium lactate, from Mallinckrodt; and dl alanine, from Eastman Kodak.

‡ Slow oxidation of d(+)xylose in the presence of glucose oxidase has been described(8).

from stock cultures were inoculated into a 200 ml capacity serum dilution bottle containing 10 ml of 20% inactivated human serum in Eagle's basal medium(9). The bottle was incubated at 36°C with nutrient renewal every second day until a confluent sheet of polyhedral cells was formed; such bottles had been found to contain about 4 or 5 million cells. After 3 successive washings with the basal medium, the cells were then nourished in the basal carbohydrate-free medium plus a test compound at 5 mM concentration. This medium was renewed every 2-4 days until the appearance of advanced degenerative changes, and then, every 7 to 14 days. Serum collected from one single horse and dialyzed extensively was used exclusively in preparing the basal medium. If variants failed to develop in 60 days, the experiment was terminated. This method is based on the principles developed in the isolation of bacterial mutants(10).

Results. When the conjunctival or HeLa cells were nourished with the basal medium plus d(+)xylose at 5 mM concentration, progressive degeneration appeared on the 4th to 7th day. In certain experiments, several small colonies of cells were observed on about the 20th to 30th day. These colonies consisted of several polyhedral, an occasional mitotic and many rounded granular cells. Gradual enlargement of these colonies with more polyhedral, more mitotic and less rounded granular cells was observed on further examination at regular intervals. Eventually, stable strains of variants capable of continuous propagation with d(+)xylose as the sole source of added carbohydrate were obtained. All the 3 xylose preparations, with or without pretreatment with glucose oxidase, were capable of supporting the propagation of these variants.

This pattern of apparent degeneration followed by regeneration was also studied quantitatively by repeating similar experiments with HeLa cells grown in a series of culture tubes. When advanced degeneration appeared in all tubes, the average number of cells per tube was calculated from results of cell counts of 2 tubes picked at random. The remaining tubes were observed at regular intervals for the appearance of variants. In a successful experiment, areas of polyhedral, mitotic and degenerating cells were observed on about the 42nd day in 1 of the remaining 4 tubes. After about 7 more days, when sufficient polyhedral cells for actual cell count were observed, the cells were trypsinized, enumerated and reinoculated into fresh xylose medium. This procedure was repeated at intervals of 1 to 2 weeks. Results are shown in Table I.

TABLE I. Growth Pattern of HeLa Cells in Xylose⁻ Medium.

Tube	No. of cells × 10^3/tube on the following days: 0*	5th†	50th	57th	70th
A	20	9	0		
B	20	9	0		
C	20	9	0		
D	20	9	34	23	138

* Based on cell number in inoculum.

† Based on avg of 2 tubes picked at random during advanced degeneration; such tubes were discarded.

The development of d-ribose variants and sodium lactate variants is in general similar to that of d(+)xylose. As of this date, we have isolated one variant capable of continuous propagation in d-ribose and one in sodium lactate from the HeLa but none from the conjunctival cells. There is suggestive evidence that d-ribose and sodium lactate variants appeared less frequently than d(+)xylose variant from our cultures of cell at this time under the described experimental conditions.

We have been unsuccessful, to date, in isolating variants from either cells capable of continuous propagation in the carbohydrate-free medium, in d-arabinose or in sodium pyruvate plus dl alanine.

Discussion. A previous communication(7) described *in vitro* propagation of these cells in various carbohydrate media. The minimal concentration of glucose capable of supporting growth, under those experimental conditions, was about 0.1 mM. With the addition of pyruvate and alanine, this concentration could be further reduced to 0.01 mM. Among the monosaccharides and intermediates tested, d-mannose, d(+)galactose and d-fructose were the only effective substitutes. It was then postulated that at least 2 μg of glucose per ml must be supplied under those conditions for the synthesis of certain essential cell components. One is tempted to hypothesize that,

while the cells from stock cultures were unable, the variants were capable of converting sufficient ribose, xylose or lactate into glucose to meet the requirements of cell growth.

It should be pointed out that the basal carbohydrate-free medium is of unknown chemical composition, on account of the presence of dialyzed serum. However, since serum from one single horse or, in critical experiments, single batch of dialyzed serum from one single bleeding was used, this variability of nutrient media composition has been reduced.

I would like to emphasize that the described experiments were not designed to study quantitative differences. The apparent frequent appearance of d(+)xylose variants may or may not occur under other experimental conditions. The successful isolation, as of this date, of d-ribose and sodium lactate variants from HeLa and not conjunctival cells may or may not be due to difference between the two cell lines. Finally, the failure to isolate d-arabinose or sodium pyruvate variants does not imply that such variants cannot be eventually isolated.

Summary. A method used successfully in isolation of nutritional variants from the human conjunctival and HeLa cells has been described. Variants capable of continuous propagation, under prescribed experimental conditions, with d(+)xylose, d-ribose or sodium lactate as the sole source of added carbohydrate or intermediate have been isolated.

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Selective Accumulation of Cd^{115} by Cortex of Rat Kidney.* (23619)

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Up to the present time the only elements with sufficiently specific localization in an organ to warrant use of their radioactive isotopes as physiological research tools have been iodine, which is concentrated in the thyroid, and zinc, which is concentrated in the rat dorsolateral prostate(1). This study using radioisotopes reports another element, cadmium, which selectively accumulates in the cortex of rat kidney.

Procedure. For the study of Cd^{115} distribution in the tissues of the rat, 40 male and female Wistar rats, 12-16 weeks of age, were used. Cd^{115} (half-life 43 days) was administered by intracardiac injection to etherized rats in doses of 8 μc/kg.† The animals were sacrificed by chloroform and exsanguination at various intervals from 1 hour to 8 months after injection. The tissues to be studied were removed from the animal, dried, weighed, and

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† Cd-115-P processed, high specific activity, was purchased from Union Carbide Nuclear Co. as $Cd(NO_3)_2$ in HNO_3 solution. Dilutions were made with physiological saline.

the radioactivity of each organ was measured by an end-window Geiger-Müller counting tube according to technics described previously(2). Studies were undertaken to determine what factors, if any, would affect the selective accumulation of cadmium in the kidney cortex. 1) *Age.* Cd^{115} was administered intraperitoneally to a total of 53 male and female rats varying in ages from 1 week to 16 weeks.‡ Twenty-four hours after injection the Cd^{115} content of kidney and liver was determined. 2) *Unilateral nephrectomy.* Unilateral nephrectomy was performed in 8 rats of varying ages from 2 weeks to 16 weeks. Two weeks post-operative Cd^{115} was administered and 24 hours later the Cd^{115} uptake was measured in the remaining hypertrophied kidney. 3) *Testosterone administration.* Administration of testosterone propionate in doses of 100 μg daily was begun in five 2-week-old male and five 2-week-old female rats. After 7 days of hormone administration the animals were injected with Cd^{115} and 24 hours later the Cd^{115} uptake was measured in kidney and liver. 4) *Orchiectomy and hypophysectomy.* Castration was performed in three 16-week-old male rats and hypophysectomy in two 16-week-old males. Cd^{115} was administered at 10, 20 and 30 days post-castration and at 10 and 20 days post-hypophysectomy. Twenty-four hours later the Cd^{115} content was measured in the kidney.

Results. Tissue distribution studies. Fig. 1 shows the gradual accumulation of Cd^{115} in the kidney cortex of the rat. The initial high concentration of Cd^{115} in the liver fell at about 20 days post-injection and was subsequently lower than cortical levels. By 5 months the kidney cortex contained approximately 2.4 times as much Cd^{115} per mg dry wt of tissue as the liver. The differences between the mean values for kidney cortex and liver at 5 months were shown to be statistically significant ($P<.01$). By 8 months post-injection the kidney cortex had accumulated approximately 4-5 times as much radio-

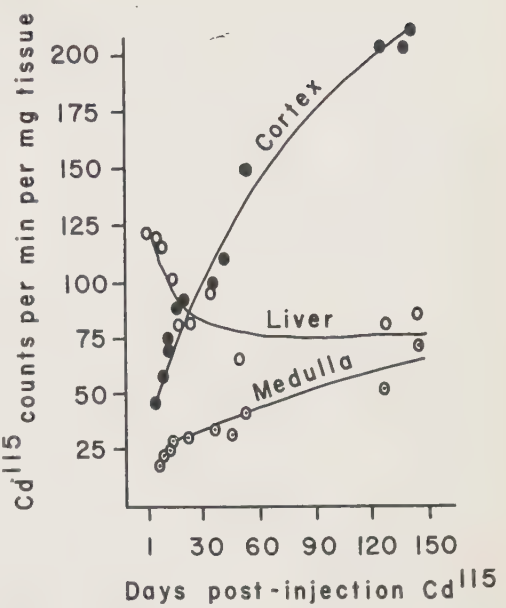

FIG. 1. Accumulation of Cd^{115} by kidney cortex of adult rat.

active cadmium per mg tissue as the liver (results not shown in the figure). Kidney medulla, though considerably lower in Cd^{115} uptake than kidney cortex, showed a gradual increase in the isotope content. By 8 months the Cd^{115} concentration in the medulla had exceeded that of the liver. The distribution of Cd^{115} in all body tissues was determined at 1, 3 and 5 hours; 1, 5, 7 and 9 days. At 1 hour the initial Cd^{115} uptake per mg of tissue was greatest in the liver. Pancreas, esophagus, ileum, large intestine and uterus contained from 30-60% as much Cd^{115} per mg as the liver. After 1 hour the Cd^{115} content of all tissues with the exception of kidney and liver, gradually fell or was maintained at low levels. By 9 days post-injection only the liver contained more Cd^{115} per mg than kidney cortex. The distribution of Cd^{115} in all other body tissues in relation to that in kidney cortex at 9 days was as follows: a) pancreas and uterus contained approximately 30% as much Cd^{115} per mg as the kidney cortex, b) seminal vesicles, coagulating gland, vas deferens, ventral prostate, dorsolateral prostate, ovary, submaxillary gland, lacrimal gland, pituitary and blood vessel contained 15-23% as much Cd^{115} per mg as the kidney cortex, c) esophagus,

‡ Studies had shown no difference in the 24-hour tissue distribution of Cd^{115} when the intraperitoneal rather than the intracardiac route of administration was employed.

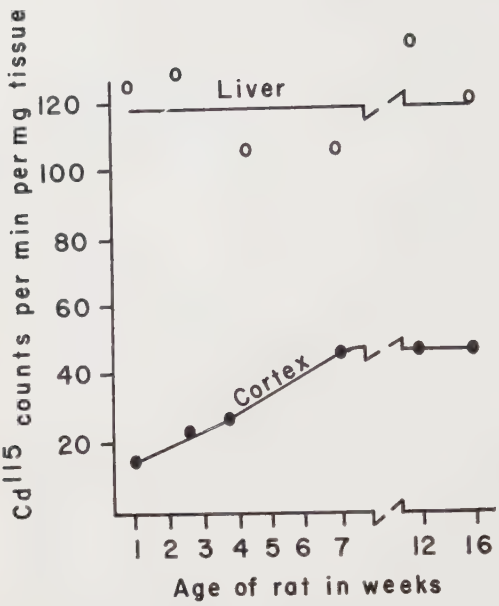

FIG. 2. Effect of age on 24-hour uptake of Cd^{115} by kidney cortex.

stomach, ileum, jejunum, large intestine, bladder, adrenal, thymus, lymph node and spleen contained 8-14% as much Cd^{115} per mg as the kidney cortex, and d) thyroid, testis, epididymis, lactating mammary gland, trachea, lung, heart, skeletal muscle, tongue, brain, spinal cord, eye, adipose tissue, skin, bone, tooth and blood contained 0-6% as much Cd^{115} per mg as the kidney cortex. Metabolic studies demonstrated Cd^{115} in the feces, the highest concentrations evident during the first 48-72 hours post-injection. Radioactivity in urine samples was absent. These metabolic findings are in accord with those recently reported by Decker and Byerrum(3).

Influence of age on Cd^{115} uptake in kidney cortex. Fig. 2 demonstrates relative inability of the young rat to concentrate Cd^{115} in the kidney cortex. With increasing age, up to about 7 weeks, there is a gradual increase in the Cd^{115} uptake per mg of tissue by the cortex. The differences between the mean values for 2½-week-old and 7-week-old rats were shown to be statistically significant ($P<.01$). The Cd^{115} uptake by the liver showed no significant differences from one age to another.

Influence of unilateral nephrectomy on Cd^{115} uptake of remaining kidney cortex. The 24-hour uptake of Cd^{115} per mg of tissue in the hypertrophied kidney following unilateral nephrectomy did not differ significantly from the intact control.

Influence of hormones on Cd^{115} uptake of kidney cortex. Neither orchiectomy, hypophysectomy, nor the administration of testosterone to the intact rat had any significant effect on the 24-hour uptake of Cd^{115} by the kidney cortex.

Discussion. One must consider whether the selective accumulation of Cd^{115} by the kidney cortex of the rat represents solely a toxic deposition of the element, or whether a part of this high concentration is a reflection of the natural amount of cadmium in the cortex which serves a definite physiological function. Evidence for a high natural cadmium content in the kidney as a whole is indicated in a report on trace elements in human tissue(4). Spectrographic analyses demonstrated that kidney contains higher concentrations of cadmium than any other tissue. The organs analyzed were from cases of accidental death, not necessarily from workers in industries using cadmium.

The studies reported in this paper revealed the inability of the renal cortex of the newborn rat to take up Cd^{115}, although age did not influence the concentration of the element in the liver. There was a gradual increase in Cd^{115} uptake by the cortex of the kidney with increasing age, reaching maximal adult levels at approximately 7 weeks of age. Kittelson (5) and Arataki(6) have reported that the cortex of the newborn rat has only one-third the number of glomeruli found in the adult, and that the full amount of glomeruli is not present until 3-8 weeks. Thus it appears that Cd^{115} uptake in the young rat kidney cortex parallels the number of nephron units present.

Studies in this paper have also shown that the 24-hour Cd^{115} uptake by the kidney cortex remains constant, in spite of unilateral nephrectomy and hormone imbalances known to affect other renal functions. This seems to answer the question raised by Shannon(7) and Taggart(8) who feel it is necessary to demonstrate unknown cellular elements available in constant but limited quantities in order to explain the known active transport of

large numbers of compounds across the tubular epithelium. Most recently Margoshes and Vallee(9) have reported the isolation of a cadmium-containing protein from the kidney cortex of the horse, thus furnishing more evidence that the presence of cadmium in the renal cortex reflects a biological need. It may be postulated that a possible function of cadmium in the kidney is concerned with water reabsorption, since mercury, a closely related element in physico-chemical properties, is known to inhibit water reabsorption in nontoxic doses.

Summary. Using radioisotopes another element, cadmium, has been found to selectively accumulate in one tissue, the kidney cortex of the rat, in concentrations sufficient to warrant its use as a research tool. The cortex of the young rat, which contains only one-third the number of nephron units found in the adult, is unable to concentrate Cd^{115} at the adult level. The studies reported indicate a possible physiological role of cadmium in renal function.

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Differentiation of Antiheparin and Thromboplastin-Generating Factors in Human Platelets.* (23620)

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Several authors(1,2,3,4) have suggested that platelets contain a factor which antagonized the effect of heparin in delaying blood coagulation. It has also been suggested that this factor is not the same as the thromboplastin-generating factor (Factor 3, of Seegers) on the basis of differential ultracentrifugation.

Materials. Platelet factors. Lyophilized human platelet material (LPM), prepared by the method of Klein *et al.*(5) was extracted with alcohol-ether (3:1 v/v) and the thromboplastin-generating material precipitated by adding three volumes of acetone in the cold. The precipitate was suspended in a small volume of water and lyophilized. This lyophilized platelet material (platelet lipid) was suspended in imidazole buffer pH 7.4 to a concentration of 1.0 mg/ml. This is the platelet lipid extract containing thromboplastin-generating activity(6). The residue of platelets after lipid extraction (lipid extracted platelet material) was freed of organic solvents and resuspended in imidazole buffer to a concentration of 3 mg/ml unless otherwise stated. *Brain lipid extract.* This was the alcohol-ether soluble acetone precipitated cephalin fraction of beef brain(7). Commercially available heparin sodium,‡ bovine fibrinogen (Fraction I§), and Thrombin Topical‖ were used.

Methods. The different materials were

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§ Armour and Co., Chicago, Ill.

‖ Parke Davis and Co., Detroit, Mich.

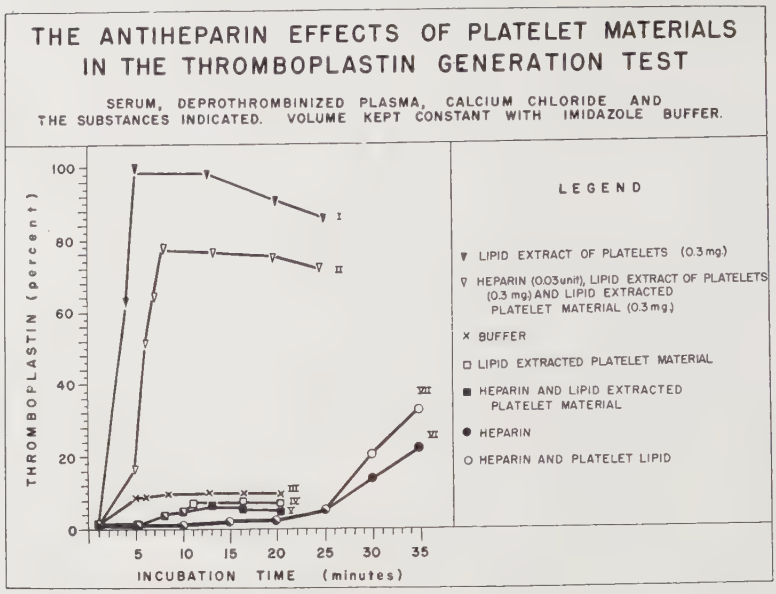

FIG. 1. Antiheparin effects of platelet materials in the thromboplastin generation test. .3 cc serum (dil. 1:10). .3 cc deprothrombinized plasma (dil. 1:5). .3 cc $CaCl_2$. .1 cc respectively of: (a) lipid extract of platelets (.3 mg/cc) of imidazole buffer; (b) heparin (.03 unit/cc); (c) LPM (.3 mg/cc of imidazole buffer). Volume kept constant at 1.2 cc with imidazole buffer.

tested in several systems to determine their effects on activity of heparin at different stages in the coagulation process. These included a simplification(8) of the thromboplastin-generation test(9), thrombin clotting times with plasma and fibrinogen(10), and recalcification times(11). To show the general property of platelets in removing anticoagulant activity from a solution of heparin, 6 mg of LPM were added to 1 cc of imidazole buffer containing 0.1 unit of heparin. The mixture was allowed to stand at room temperature for 5 minutes, then centrifuged at 30,000 g for one hour. The supernatant was used in the thromboplastin-generation test in comparison with the sediment, with the same solution of heparin, and with a suspension of platelets in buffer treated similarly. Lipid-extracted LPM was also tested in the same way. The thromboplastin-generation test was used to investigate the antiheparin property of LPM as compared to fresh platelets in more detail, as follows: first, 0.1 ml of 0.3 unit/ml of heparin and of heparin incubated with LPM, were added to the incubation mixture at the beginning of the experiment to examine its effect on thromboplastin production. Various amounts of heparin were used in the thrombin and recalcification times according to the sensitivity to heparin of the plasma employed.

Results. In the simple test of the property of platelets to remove heparin activity from a solution, it was found that 6 mg of LPM or lipid-extracted platelets completely neutralized the effects of 0.1 unit of heparin, similar to equivalent amounts of fresh platelets. The results of the more detailed studies are shown in Fig. 1 and 2.

(1) *Thromboplastin-generation test.* Fig. 1 shows the results of a representative system of tests expressed as percentage of thromboplastin formed at different periods of incubation. Comparison of the curves shows the following: thromboplastin was rapidly generated when the lipid extracted from platelets was used (Curve I). Only a small amount of thromboplastin was very slowly formed when heparin (0.03 unit) was added to this system (Curve VII). Formation of thromboplastin was restored to nearly normal by the addition of lipid-extracted platelet material to the system (Curve II). This lipid-extracted material did not, itself, have any thromboplastin-gen-

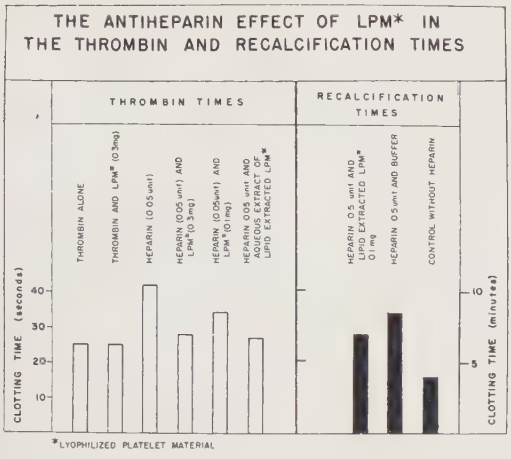

FIG. 2.

erating potency, the curve obtained when it, alone, was added to the system (Curve IV) being similar to that obtained when buffer was used (Curve III). However, when used alone, the lipid-extracted platelet material was partially effective in neutralizing heparin in the earlier stages of incubation (Curve V compared with Curves VI and III). Similar results were obtained when brain lipid extract was used in place of the platelet lipid in these experiments. A combination of brain lipid extract and lipid-extracted platelets reversed the effects of heparin. Whole platelets have the same antiheparin effect as the combination of brain lipid and lipid-extracted platelet material. Increasing the concentration of brain lipid extract did not, by itself, reverse the action of the heparin.

(2) *Thrombin clotting times.* Results in these tests are shown in Fig. 2, and demonstrate phenomena essentially similar to those observed in the thromboplastin-generation test. It will be seen that platelet materials do not have any accelerating effect on the thrombin time alone, but that in suitable concentrations they are able to antagonize the effect of heparin on it. Similar results were obtained in studying the reversibility of the effects of heparin on the clotting time of recalcified plasma. The corresponding data in Fig. 2 represent the averages from over 200 individual determinations on the plasma samples obtained from 58 normal donors.

Summary. Lyophilized human platelet material can be separated into thromboplastin-generating and heparin-antagonizing fractions. The heparin-antagonizing fraction does not, in itself, possess any thromboplastic activity but it reverses the effects of heparin on thromboplastin formation and thrombin activity. The material responsible is present in an aqueous extract of lipid-extracted platelets.

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Effect of Plasma from Bled and Phenylhydrazine Treated Animals on Plasm Iron Turnover. A Test for "Hemopoietine". (23621)

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Experiments carried out in several laboratories point to the existence of a factor capable of stimulating erythropoiesis, "Hemopoietine" of Carnot and Deflandre(1), in plasma of animals in which red cell formation has been increased by bleeding(2), phenylhydrazine(3), and exposure to low barometric pressures(4). In working with this factor it is necessary to have an adequate, rapid biologic test for assaying its activity. The majority of workers give several injections of plasma, or plasma extracts from anemic animals, usually phenylhydrazine treated rabbits, to normal rats, and observe the increase in one or all of the following: Hb concentration, hematocrit, red cell and reticulocyte counts. Some workers obtained increase in Hb concentration(3,5) others only in red cell count with no increase in Hb and hematocrit(6). The reticulocyte responses observed in some experiments are quite striking, in others not at all clear cut. These tests are time consuming, use indirect methods for measuring erythropoiesis and raise the question of validity of utilizing another species, the rat, as an assay for activity of an erythropoietic factor, possibly a mucoprotein(7) obtained from the rabbit. Recent studies with Fe^{59}(8) have shown that the plasma iron turnover is a good index of Hb synthesis. It was therefore decided to investigate the effect of plasma and plasma extracts of anemic rabbits, on plasma iron turnover in normal rabbits, with a view to developing a useful biologic test for study of the properties of the erythropoietic factor (or factors) "Hemopoietine," present in the blood of animals with increased erythropoiesis.

Material and methods. Rabbits of both sexes, average weight 2100 g (1600-2400), were used as experimental animals. The rabbits, kept in individual cages and given a standard diet obtained from the San Cristobal Milling Co., Santiago, Chile, were divided into groups of donor and receptor animals. As donors, normal rabbits, bled rabbits and rabbits treated with phenylhydrazine were used. The receptors were normal rabbits. The plasma from bled rabbits was obtained on the 4th or 5th day after daily bleedings of 15-20 ml/kg when Hb levels were about 5 g%. The phenylhydrazine treated animals were exsanguinated after 6 days of treatment with 7.5 mg/kg daily of this drug. Average Hb concentration at the time of bleeding was 4 g%. Heparin was used throughout the experiments as anticoagulant. In some experiments plasma was injected in the native state; in others plasma dialyzed 24 hr against 30 volumes of .9% NaCl solution, or plasma extracts prepared according to Borsook(3) were used. In one experiment an acid acetone extract of bled rabbit's plasma was injected. In all experiments plasma, or plasma extracts were injected intraperitoneally in the receptor rabbits in doses of 10 ml/kg twice a day for 2 days. Plasma iron turnover studies were made approximately 16 hr after the last plasma injection. Two types of control experiments were run, normal untreated rabbits and rabbits injected with normal rabbit's plasma. Rabbits with over 11.5 g% Hb and less than 3% reticulocytes, in good physical condition, were used as normals. For determination of plasma iron turnover $Fe^{59}Cl_3$ of high specific activity (1-3 c/g) obtained from Oak Ridge, was used. Previous to injection the $Fe^{59}Cl_3$ was transformed into Fe^{59} ascorbate(9) and incubated for 10′ at room temperature with serum, the unsaturated iron binding capacity of which was measured previously(9). Dose of Fe^{59} injected intravenously was approximately 1 μc/rabbit; an aliquot of the injected Fe^{59} serum was diluted to 50 ml, with .9% NaCl solution and kept as a standard. Blood samples (3 ml) were obtained from animals 20′, 40′, 60′ and 80′, 24 and 48 hrs after Fe^{59} injection. Radioactivity

TABLE I. Mean Values for Plasma Iron in 10 Rabbits at 20′, 40′, 60′ and 80′ after Injection of Fe^{59}.

Time	20′	40′	60′	80′
Plasma iron, μg/ml	2.17	2.11	2.18	2.20

TABLE II. Fe^{59} Incorporation into Red Cells, 24 and 48 Hr after Injection in Normal Rabbits, in Which No Samples for Plasma Radioactivity Measurements Were Taken, and in Normal Rabbits in Which Sampling Was Done at 20′, 40′, 60′ and 80′ after Fe^{59} Injection.

Group	No. of rabbits	Fe^{59} incorporation into red cell (% inj. dose) 24 hr	48 hr
NR, no samples	5	16.4	27.0
NR samples	7	35.7	64.4

of plasma samples was measured with NaI (Tl) Scintillation Counter (Philips, Eindhoven); 3000 counts were totalized in each sample. The cpm/ml plasma *vs.* time, were plotted on semi-log paper and a line fitted to the points. The plasma iron turnover time-constant K, was calculated from the following equation: $K = \frac{0.693}{T½}$, where T½ is the plasma radioactivity half life determined graphically. Correlation analysis carried out in 25 individual experiments showed that a significant linear correlation existed between log cpm/ml plasma and time ($-r > 0.98$). In view of this, calculation of turnover time constants was carried out as indicated previously. Volume of distribution of Fe^{59}-transferrin, was calculated from the following equation: $Vd = \frac{D}{cpm/ml_{to}}$, where Vd = volume of distribution, D = dose of Fe^{59} injected, expressed as cpm and cpm/ml_{to} = cpm/ml at zero time, as obtained from graphic extrapolation, of the line relating log cpm to time. Plasma iron concentration was determined by the method of Ramsay(10). In preliminary experiments, plasma iron determinations were carried out in each of the samples drawn at 20′, 40′, 60′ and 80′. It was seen from these experiments that the fluctuation of plasma iron levels during the period of sampling was not marked (Table II). In view of this, iron determinations for plasma iron turnover calculations were made using pooled plasma from the 4 blood samples. With reference to accuracy of the method, duplicate determinations of plasma iron showed a maximum variation of .2 μg/ml and a series of 10 determinations of iron, in a standard solution of iron containing 2 μg/ml, gave a coefficient of variation of 2.9%. Plasma iron turnover was calculated from total plasma iron, multiplied by K (the turnover constant). Fe^{59} incorporation into red cells was also measured in these experiments. For calculation of percentage of Fe^{59} appearing in the circulating red cells, the blood volume of the rabbit was taken to be 57 ml/kg. This value was obtained by us in measurements carried out in 20 normal rabbits, using Evans Blue and P^{32} labelled red cells. Percentages of Fe^{59} incorporation into red cells are included for purposes of illustration, but do not have quantitative value, since we have observed that the blood sampling of 3 ml at 20′, 40′, 60′, and 80′ for plasma radioactivity measurements, markedly influences the incorporation of Fe^{59} into red cells (Table I). However, the Fe^{59} uptake measurements are useful in that they show the trend in the different experiments in which plasma samples were taken.

Results. The results are summarized in Table III in which the following abbreviations have been used. NR = normal rabbits. NRP = normal rabbits' plasma. BRP = bled rabbits' plasma. BRDP = bled rabbits' dialyzed plasma. Acet BRP = acetone extract of bled rabbits' plasma. PhRP = phenylhydrazine treated rabbits' plasma. PhRBP = phenylhydrazine treated rabbits' boiled plasma. Table III shows values for plasma iron turnover and red cell incorporation of Fe^{59} in the above mentioned groups. Plasma from phenylhydrazine treated rabbits markedly increases plasma iron turnover, while plasma from bled rabbits, although producing a significant increase in the percent per hour turnover, does not increase plasma iron turnover, since it produces a marked drop in plasma iron: 208 to 126 μg bled rabbits' plasma after dialysis does however increase plasma iron turnover and its effect on plasma iron is less marked. Normal rabbits' plasma

TABLE III. Plasma Iron Turnover and Fe^{59} Incorporation into Red Cells in Normal Rabbits (NR) Injected with: Normal Rabbits' Plasma (NRP), Bled Rabbits' Plasma (BRP), Bled Rabbits' Dialyzed Plasma (BRDP), Acetone Extract of Bled Rabbits' Plasma (Acet BRP), Phenylhydrazine Treated Rabbits' Plasma (PhRP) and Boiled Plasma of Phenylhydrazine Treated Rabbits (PhRBP). Values indicated are means and standard errors. Mean values for plasma iron turnover are not calculated from the product of the mean values of plasma iron and the iron turnover time constant, but are means of the individually calculated plasma iron turnover.

Group	No. of rabbits	Plasma iron turnover time constant (%/hr^{-1})	Total plasma iron (μg)	Plasma iron turnover (μg/hr^{-1})	Fe^{59} incorporated into red cells (% doses) 24 hr	48 hr
NR	8	58.0 ± 4.54	208.0 ± 18.8	116.0 ± 13.9	35.7 ± 3.14	64.4 ± 3.1
NRP	10	61.1 ± 4.4	182.0 ± 13.3	108.0 ± 9.25	54.0 ± 2.92	80.5 ± 1.85
BRP	13	99.5 ± 3.27	126.0 ± 9.0	124.6 ± 8.77	53.6 ± 1.85	91.6 ± 1.41
BRDP	9	114.0 ± 6.8	144.0 ± 10.86	157.0 ± 8.26		95.2 ± 1.92
Acet BRP	4	59.5 ± 4.07	90.5 ± 6.52	53.5 ± 4.55		73.5 ± 4.22 (72 hr)
PhRP	15	117.0 ± 6.07	224.2 ± 15.63	260.0 ± 17.86	72.3 ± 3.5	74.7 ± 5.11 (72 hr)
PhRBP	9	159.0 ± 9.4	163.0 ± 23.7	272.0 ± 37.12	68.4 ± 3.5	84.4 ± 4.61

does not affect plasma iron turnover. With reference to the values for Fe^{59} incorporation into red cells the trend is for all plasma injections to produce an increase in Fe^{59} uptake. PhRP produces a maximum uptake at 24 hr, while NRP and BRP show maximum uptake at 48 hr, with the BRP group showing larger uptake at 48 hr than the NRP group. The supernatant obtained after boiling plasma from PhRP at pH 5.5 produces a marked increase in plasma iron turnover in spite of the fact that the plasma iron is slightly lowered. On the other hand, the acid acetone extract of bled rabbits' plasma notably lowers plasma iron turnover and plasma iron.

Discussion. Plasma from phenylhydrazine treated rabbits produces a clear cut increase in plasma iron turnover and in the rate of incorporation of Fe^{59} into red cells, when injected into normal rabbits. These probably reflect an increased Hb synthesis in these animals. Plasma from bled animals does not increase plasma iron turnover for although it markedly increases the speed of Fe^{59} disappearance, it also lowers plasma iron. The finding emphasizes the importance of measuring plasma iron in this type of study. The experiments with dialyzed bled rabbits' plasma and with acetone extracts of this plasma point to the existence in the blood of severely bled rabbits of at least 2 factors influencing iron metabolism: one nondialyzable which increases iron turnover and another dialyzable, soluble in acid acetone, which lowers plasma iron concentration and plasma iron turnover.

The finding that boiling does not inactivate PhRP disagrees with experiments of Erslev (11) but accords with the results of Borsook *et al.*(3) Gordon, *et al.*(5) and Linman and Bethell(6). However it is important to point out that the conclusions of the latter, who suggest that the factor is probably nonprotein, are open to doubt, as we have observed that the supernatant from boiled plasma treated with perchloric acid, contains a significant amount of phosphotungstic acid precipitable material which reacts with Biuret and has a carbohydrate content of around 4 mg/100 mg protein (seromucoid).

The percentage of the injected Fe^{59} appearing in the red cell at 24 and 48 hr is a function of many variables: size of plasma labile iron pools, uptake of iron by the erythroblasts and rate of release of red cells containing labelled hemoglobin from the marrow. Thus the index of Fe^{59} uptake when used alone, does not necessarily indicate the state of erythropoiesis. This point is illustrated by the results of our experiment with Acet BRP and PhRP, in which the 48 hr Fe^{59} uptakes are similar and yet plasma iron turnover is 5 times smaller in one group than in the other.

Conclusions. 1) Plasma obtained from phe-

nylhydrazine treated rabbits markedly increases plasma iron turnover. The supernatant obtained after boiling this plasma retains its effect on iron turnover. 2) Bled rabbits' plasma does not affect iron turnover, but has a clear cut lowering effect on plasma iron. Dialyzed plasma of bled rabbits, however, increases plasma iron turnover, while acetone extracts of bled rabbits' plasma, significantly depress plasma iron and plasma iron turnover. 3) The fraction of Fe^{59} appearing in the red cells at 24 and 48 hr is increased by normal plasma, bled rabbits' plasma, phenylhydrazine treated rabbits' plasma and is not a good index of the state of erythropoiesis. 4) Analysis of the effect of anemic rabbits' plasma on iron metabolism as studied with tracer doses of Fe^{59} gives information on the factors controlling iron metabolism and presents a practical method for bioassay of the plasma erythropoietic factor "Hemopoietine."

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Further Studies on Diuresis in Normal and Adrenalectomized Rats Following Total-Body X-Irradiation.* (23622)

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Experiments on changes in water balance in relation to total-body irradiation have been carried out by France(1) and Edelmann(2,3) and in relation to partial-body irradiation as well by Smith and Tyree(4). Both polydipsia(4) and polyuria(5), which occur in rats following total-body exposure, have been shown to be dependent upon the presence of the adrenal cortex. Removal of the anterior pituitary prior to irradiation eliminates polyuria, but removal of the posterior pituitary (5) does not. Certain irregularities appear in the literature concerning changes in water balance following irradiation. For example, Smith and Tyree(4) found that only a certain percentage of rats manifested polydipsia following irradiation. This percentage could be increased by increasing the x-radiation dosage. Also, Kay and Entenman(6) report that irradiation of rats 1, 3, and 7 days following adrenalectomy does not prevent diuresis.† In view of these and other inconsistencies, it was believed that the influence of additional factors in relation to water balance and irradiation should be examined.

Factors that might be relevant were the state of hydration of the animals at the time of exposure, water intake following irradiation, body weight, and length of time that elapsed between adrenalectomy and x-irradiation. Olewine and Perlmutt(7) were able to show that while irregularities in potassium ex-

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† Personal communication.

cretion were evident as soon as 3 days following adrenalectomy in rats, excess of sodium excretion did not appear for 3 weeks. It would therefore seem that some time must elapse for animals to reach a new state of equilibrium following removal of adrenals.

In some of our own unpublished experiments, increase in urine excretion following total-body irradiation bore a linear relationship to increasing doses of x-ray, with the smallest effective dose being somewhere between 50 and 100 r. Other unpublished experiments yielded results that did not show the above relationship. The following experiments were designed in an effort to elucidate some of the factors pertinent to the observed and reported variation of results.

Methods. Female Sprague-Dawley rats were used and were housed at a temperature of 78°F ± 2°. Except during urine collection periods, they were given Rockland mouse pellets and water *ad libitum.* Adrenalectomized animals were given 1% sodium chloride as drinking water. Rats were grouped by randomizing on a body weight basis. Collection of urine free of extraneous material was accomplished by placing the animals in metabolism cages‡ (2 per cage) for 6 hours during the day, during which time they were fasted but had free access to drinking fluid.§ A period of training to this regimen of at least 4 days always preceded irradiation unless otherwise noted. Animals continue to gain in body weight under these conditions. Urine was collected in 50 ml graduates placed under the collection tubes of the metabolism cages. Volumes recorded in the tables represent ml of urine per rat per 6 hour period. Irradiation was carried out early in the morning and the first subsequent urine collection period was the 6 hours immediately following. Adrenalectomy was performed by bilateral incisions, and all animals were examined at autopsy for the presence of residual or regenerated adrenal cortical tissue. Where there was any question tissues were taken for histological examinations and any animals proving to have what appeared to be functional tissue were deleted from the series. Irradiation was achieved by placing unanesthetized rats in individual sections of a circular aluminum carrier that was perforated uniformly for maximum exposure. This carrier was placed on a slowly rotating table during irradiation. The radiation was generated by a 250-KVP Maxitron machine operating at 30 ma. The target-to-skin distance was 92 cm, and exposure rate was approximately 30 r per minute. Control nonirradiated animals were subjected to the same manipulations.

Results. To test the diuretic response in animals of varying body weights to total-body irradiation the following experiments were carried out: Groups of 6 animals each were maintained in feeding cages and their water supply was supplemented by placing open bowls of water within the cages. In Exp. 1 the response to 300 r was tested. Irradiation was preceded by a 4-day training period to the fast, and change in urine output for the 6-hour collection period was calculated by taking average urine volume per rat for 3 days prior to irradiation from the average urine volume of 2 collection periods subsequent to exposure, that is on the day of x-radiation and day following. Results are shown in Table I. In Exp. 2, 400 r was used and an 8-day training period. In this experiment, water intake during the 6-hour fast was recorded as well.‖ Change in water intake and urine output were calculated by subtracting 4-day averages prior to exposure from the 2-day average following irradiation. These results are also shown in Table I. In both of these experiments, all pairs of animals on either the first or second day following irradiation and in some cases showed positive increases in urine output. Inspection of the data makes it abundantly clear

‡ Manufactured by Acme Sheet Metal Works, Chicago. An additional screen of 8 mesh, 0.027 wire was placed horizontally in the collection funnel 1 inch below floor of cage. A cylinder of similar wire screen was fitted to delivery tube of the collection funnel so that approximately 1 inch protruded above the tube. Urine so collected from healthy rats is satisfactorily free from solid material.

§ Unless otherwise stated (see text and tables).

‖ Water intake figures were calculated by placing a measured amount of water in bottles of the metabolism cages and measuring the amount which remained at the end of the 6-hour fasting period.

TABLE I. Changes in Water Intake and Urine Output of Rats of Various Body Weights following Total-body Exposure to 300 r and 400 r of X-radiation.

Exp. I			Exp. II			
X-ray dose (r)	Body wt (g)	Change in urine output (ml)	X-ray dose (r)	Body wt (g)	Change in water intake (ml)	Change in urine output (ml)
300	73	+3.2	400	110	+5.8	+2.6
0	73	+ .6	0	105	− .7	+ .2
300	125	+6.2	400	146	+4.0	+2.4
0	132	+ .5	0	148	−1.2	+ .2
300	166	+5.8	400	183	+3.0	+1.8
0	173	− .5	0	178	− .8	− .5
300	218	+4.6	400	217	+2.5	+2.1
0	206	+ .2	0	215	− .7	+ .7
300	250	+2.4	400	225	+6.0	+3.7
0	250	+1.0	0	262	+ .2	+ .1

All groups were composed of 6 animals each.

that under these experimental conditions body weight had no constant effect on the amount of urine excreted following total-body irradiation. In Exp. 2, increase in water intake was apparent in all irradiated groups. These figures do not reflect total water balance because they represent only 6 hours.

Since it was apparent both from these and the experiments of others(1,4) that polyuria is accompanied by polydipsia, the following experiments were done to establish which came first, the polydipsia or the polyuria. One author, France(1), states that there is no increase in the flow of urine when water intake is restricted, but no supporting data are given.

Exp. 3 consisted of 4 groups of 10 animals, each having an average body weight of 133 g. They were trained to the 6-hour fast for 7 days prior to irradiation. Drinking water was withheld during the first 6-hour collection period immediately following irradiation in all groups. Urine volumes are recorded for 3 consecutive collection periods in Table II.

It is evident that during the first 6-hour collection period when drinking water was withheld no diuresis occurred. On the day following, however, having had water *ad libitum* for the previous 16 hours and with water allowed during the collection period, increasing polyuria with increasing x-radiation dosage was evident. Column 6 of Table II shows the increase in urine excreted on the second post-irradiation day over that excreted during the pre-irradiation period. Each pair of animals in all irradiated groups showed an increased flow of urine on the second day. The average of these increases bears a straight line relationship to the amount of x-ray given. An additional experiment was carried out that was similar to Exp. 3 except that a 6-day training period was used and the dosage was limited to 400 r. This experiment included controls that were allowed drinking water during the first 6-hour collection period. The results are shown in Table III. Here, too, when diuresis appeared, it occurred in each pair of all irradiated animals.

The following experiment was done to test

TABLE II. Changes in Urine Volume of Rats Irradiated with Various Amounts of X-ray and Having Drinking Water Withheld during First Collection Period.

1	2	3	4	5	6	7
			Water withheld for first 6 hr			
Group	Urine vol, 4 day avg, prior to x-ray (ml)	X-ray dose (r)	Urine vol 1st 6 hr after x-ray (ml)	6 hr urine vol 2nd day after x-ray (ml)	Column 5 minus 2 (ml)	6 hr urine vol 3rd day after x-ray (ml)
I	2.2	200	1.4	4.5	2.3	3.5
II	2.0	400	1.6	6.4	4.4	3.1
III	2.5	600	2.2	9.2	6.7	1.9
IV	2.3	0	1.7	2.3	.0	3.0

All groups were composed of 10 animals each.

TABLE III. Changes in Urine Volume of Four Groups of Irradiated Rats with and without Adequate Access to Drinking Water.

Urine vol, 4 day avg, prior to x-ray (ml)	X-ray dose (r)	Urine vol 1st 6 hr after x-ray (ml)	6 hr urine vol (ml) 2nd day after x-ray	6 hr urine vol (ml) 3rd day after x-ray
		Water withheld for 1st 6 hr	Water given	
2.4	0	1.6	1.9	1.7
3.5	400	2.0	12.5	6.4
		Water given	Water given	
2.6	0	1.2	2.0	1.5
2.7	400	14.0	10.7	7.4

All groups were composed of 10 animals each.

the effect of total-body irradiation on adrenalectomized rats when they were irradiated at various time intervals following the operation. Three groups of 24 animals, each weighing 125, 95, and 65 g respectively, were caged in groups of 6, and 12 animals of each weight group were adrenalectomized. The other half of each group was sham-operated. The animals weighing 125 g were allowed 2 days to recover from surgery and then were given a 3-day training period to the 6-hour fast and were irradiated as shown in Table IV. Animals of the second weight group were given a 4-day training period to the fast and were irradiated on the 12th postoperative day. Only 8 adrenalectomized animals of the third weight group were suitable for experiment. These along with sham-operated groups were irradiated on the 22nd postoperative day following a 4-day training period. Average body weight of all the adrenalectomized groups at irradiation was 138 g. Urine excretion figures for the 4 days following irradiation are shown in Table IV.

Each pair of adrenalectomized animals that was irradiated 5 days following operation showed a 2 ml or more increase in urine excretion on the first day. In 4 of the 6 animals, the increase persisted through the second day. One animal died on the fourth day. Of the animals that were irradiated 12 days postoperatively, one pair showed an increase in urine excretion that exceeded 2 ml. No other diuresis was apparent and all animals were dead by the fourth day. None of the animals that were irradiated 22 days after adrenalectomy showed diuresis. Six out of 8 were dead on the fourth day. All of the irradiated sham-operated animals showed diuresis that usually persisted through 4 days.

Discussion. In Exp. 1 and 2 every effort was made to see that all animals had adequate access to drinking water during the course of the experiment. It had been observed that when more than 6 animals were housed in one feeding cage the total supply of water in the feeders would frequently be completely used prior to refilling. When this condition obtained much less consistent and dramatic polyuria occurred than when an abundance of water was available at all times. However, even under these conditions, no regular pattern of fluid excretion appears. The most con-

TABLE IV. Changes in Urine Volume of Adrenalectomized Rats following Total-body X-irradiation at Various Post-operative Times.

No. days between surgery and irrad.	Operation	Urine vol, 3 or 4 days avg, prior to x-ray (ml)	X-ray dose (r)	Urine vol 1st 6 hr after x-ray (ml)	6 hr urine vol (ml) 2nd day after x-ray	6 hr urine vol (ml) 3rd day after x-ray	6 hr urine vol (ml) 4th day after x-ray
5	Adrenalectomy	2.9	400	4.9	3.8	3.0	3.4
	"	2.4	0	1.1	2.4	2.3	3.0
	Sham operation	2.2	0	1.7	1.5	1.0	1.4
	" "	2.2	400	13.7	10.8	8.2	6.1
12	Adrenalectomy	3.1	0	2.2	4.0	4.6	4.1
	"	2.9	400	3.3	2.4	3.0	all dead
	Sham operation	2.0	400	11.7	7.8	3.9	4.4
	" "	2.1	0	2.0	2.5	3.0	1.5
22	Adrenalectomy	3.4(8)*	400	3.3	2.5	3.1	6 dead
	Sham operation	2.6	400	8.1	5.3	4.9	3.5
	" "	2.7	0	1.1	2.5	1.5	1.3

* No. of animals in this group. All other groups were composed of 6 animals.

sistent relationship that is evident is that the changes in water intake parallel the changes in urine output reasonably well.

The straight line relationship obtained in Exp. 3 is not quantitatively consistent from experiment to experiment. In Exp. 4, dosage of 400 r elicited twice the volume of urine. As far as could be ascertained, the physical conditions of both experiments were equivalent. The fact that a quantitative relationship between x-radiation dosage and urine output does occur sometimes suggests that a single biologic component or mechanism on which flow of urine depends is specifically responsive to irradiation. Since polyuria is dependent on the polydipsia, it is evident that x-irradiation stimulates thirst. The experiments of Gilman(8) suggest that cellular dehydration is the prime factor in arousing thirst. Since France(1) has been able to show a significant increase in the thiocyanate space in rats 2 days after a single total-body irradiation of 400 r, it seems probable that this is the mechanism that is responsible for polydipsia. It is not yet clear in exactly what manner the hormones of the adrenal cortex mediate the shift in body water. Experiments to further elucidate the above-mentioned relationships are in progress.

Summary. 1. Experiments have failed to show any influence of body-weight on the amount of urine excreted by the rat following total-body irradiation. 2. Rats that had at all times an excess of drinking water always showed an increase in the flow of urine following exposure to 400 r. 3. If water was withheld immediately following total-body irradiation, no immediate diuresis resulted. If drinking water was allowed after being withheld for 6 hours following irradiation, polyuria then followed the polydipsia. Under these conditions, a quantitative relationship sometimes obtained between the amount of x-radiation and the increase in urine excreted. 4. Following adrenalectomy, a time lapse of at least 12 days must be allowed before an obliteration of the diuretic response to total-body x-irradiation is apparent.

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Chromatophore Hormones in the Pituitary of Albino Animals. (23623)

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We have shown(1) that at least 2 chromatophore hormones exist, one a hormone of the anterior pituitary lobe and one situated in the posterior lobe. The studies of Zondek(2) and Lerner(3) as well as our own(1) indicate that as many as 3 hormones may exist. To distinguish between them we have proposed the following nomenclature: Posterior chromatophore hormone = Melanophore hormone (Herring), Intermediate chromatophore hormone = Intermedin (Zondek), and Anterior chromatophore hormone = Chromatophorotropic factor of ACTH (Sulman). It is difficult to ascribe definite functions to the various chromatophore hormones, particularly to the posterior chromatophore hormone, but it is known that the intermediate chromatophore hormone is directly concerned with albinism

since albino frogs lack the pars intermedia of the pituitary(4).

The existence of more than one chromatophore hormone justified a reexamination of the problem of melanophore activity in albinos. Bloch(5) and Schultz(6) established that albinism is characterized by the lack of DOPA oxidase. Since 1925 only few studies have been devoted to albinism. Beadle(7) and Russell *et al.*(8) confirmed the absence of DOPA oxidase, as did the research of Fitzpatrick and Lerner(9). The latter, however, proposed the use of the wider term tyrosinase in preference to DOPA oxidase, and also confirmed earlier histochemical studies (10) of albino skin which failed to demonstrate the presence of tyrosinase in the melanocytes of albinos. Meirowsky(11) stated that inheritable albinism is a recessive characteristic which is contained in the genes in a cryptomere condition. He further stated that albinism may disappear in some patients in the course of their lives due to formation of tyrosinase. A preliminary experiment conducted by us in patients suffering from vitiligo has shown that pigmentation can be partly restored to the albinotic areas by the injection of a combination of tyrosinase and melanophore hormone(12).

Technic. The pituitaries were removed from rabbits immediately after they were sacrificed. Separation of the pituitary lobes was carried out under a 10 x magnifying glass immediately after death. The separated lobes were weighed on a torsion micro-balance and homogenized in a glass tube with barrel homogenizer, by adding 0.5 ml of n/10 NaOH/10 mg tissue. The resulting emulsions were neutralized (bromthymol blue indicator) with a few drops of 1.5% acetic acid, brought to a concentration of 10 mg/ml with water and assayed for chromatophore hormone in green-adapted tree-frogs of the species *Hyla arborea*(13-15). Three frogs were used for each dose, and one pituitary lobe was titrated on at least 20 frogs. One frog unit chromatophore hormone in tree-frogs (*Hyla arborea*) is approximately equivalent to 0.1 γ active substance.

Results. Table I shows that the chromato-

TABLE I. Chromatophore Hormone Content of Anterior + Intermediate and Posterior Lobes of Rabbit Pituitaries.

Color of rabbit	Rabbit wt, g	Pituitary lobe	Wt of lobe, mg	Homogenate (10 mg/ml) dilution 1:1 .3 ml	1:1 .1 ml	1:10 .3 ml	1:10 .1 ml	1:100 .3 ml	1:100 .1 ml	1:1000 .3 ml	1:1000 .1 ml	Chromatophore hormone, frog units/lobe
Grey	1550	Ant. + int.	12.5	+++	+++	+	±	—	—	—	—	38
		Posterior	5	+++	+++	+	+	+	±	—	±	150
"	1600	Ant. + int.	11	+++	+++	+	±	—	—	—	—	33
		Posterior	3	+++	+++	+	+	+	—	—	—	90
"	1700	Ant. + int.	14	+++	+++	+++	+	±	—	—	±	140
		Posterior	5	+++	+++	+	+	+	±	±	—	150
"	1650	Ant. + int.	10	+++	+++	+++	+	±	—	±	—	100
		Posterior	3	+++	+++	+	+	+	±	—	—	90
"	1500	Ant. + int.	13	+++	+++	+	+++	—	—	—	—	130
		Posterior	5	+++	+++	+++	+	±	—	±	—	50
"	1750	Ant. + int.	15	+++	+++	+	+	±	±	±	—	150
		Posterior	5	+++	+++	+	±	±	—	—	—	15
Black	1700	Ant. + int.	13.5	+++	+++	+	+	—	—	—	—	135
		Posterior	4	+++	+++	+	+	±	—	±	—	40
Albino	1700	Ant. + int.	14	+++	+++	+	±	—	—	—	—	42
		Posterior	5	+++	+++	+++	+	+	±	±	—	150
"	1650	Ant. + int.	18	+++	+++	+	±	±	—	—	—	54
		Posterior	5	+++	+++	+	±	—	—	—	—	15
"	1700	Ant. + int.	14	+++	+++	+++	+++	+	±	±	±	42
		Posterior	4	+++	+++	+++	+++	±	±	—	—	40
"	1750	Ant. + int.	15	+++	+++	+++	+	—	±	—	—	150
		Posterior	5	+++	+++	+	+	+	+	±	—	500

phore hormone content in pigmented rabbits varies in the anterior + intermediate lobes between 33-150 frog units and in the posterior lobes between 15-150 frog units. In albino rabbits the values found amounted to 42-150 frog units in the anterior + intermediate lobes and to 15-500 frog units in the posterior lobes. Thus it is evident that the albino animal produces chromatophore hormone at least to the same extent as the normally pigmented rabbit and there is no significant difference in the pituitary content of this hormone between either type of animal.

Summary. The chromatophore hormone content of the pituitaries of pigmented and of albinotic rabbits has been examined in tree frogs (*Hyla arborea*). There is no significant difference between the two types of animals, the hormone content in the anterior + intermediate lobes in pigmented animals amounting to 30-150 frog units and in albinotic animals to 42-150 frog units. In the posterior lobes the values for pigmented animals were between 15 and 150 frog units and for albinotic animals between 15 and 500 frog units. We conclude that the only difference between the albinotic and the normally pigmented individual is the lack of enzymes of the type of DOPA oxidase or tyrosinase. This fact should be borne in mind in the approach to the treatment of vitiligo.

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Effect of Large Loads of Sodium on Bone and Soft Tissue Composition.* (23624)

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Recent data from this laboratory have demonstrated that in acute sodium depletion, approximately 70% of the sodium removed from the body is derived from the extracellular phase, 25% from bone mineral, and only 5% from the intracellular compartment(1). In view of these findings it was decided to determine whether bone could also act as an acceptor of sodium ions, under conditions of prolonged sodium-loading, or whether excess sodium would be retained in the extracellular fluid or the body cells. Therefore, a small group of rats was given a daily sodium load approximating 50% of their total body sodium. After 24 days they were sacrificed and the sodium content of bones and soft tissues determined. It was found that this procedure caused an increase of 8.6% in bone mineral sodium, associated with a 24% loss of bone water. These changes in bone occurred in the absence of changes in body weight, extracellular fluid composition, or the composition

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of soft tissues as exemplified by muscle and liver.

Methods and calculations. Two groups of 400 g male albino rats (3 control, 5 experimental) were placed on a diet of Purina rat checkers, with fluid *ad lib.*, for a period of 24 days. The rats were of identical age, one year and one week, and had been raised under identical conditions for 10 months preceding the experiment. Their weight had been stable for 2 months. These precautions were observed so as to eliminate from the experiment those changes in bone water and sodium which are known to occur during growth(2). The experimental rats received 0.85% sodium chloride as drinking water, and in addition were given 1 ml of 3% sodium chloride/100 g of body weight, intraperitoneally, 5 days each week for a total of 19 doses. At sacrifice, the animals were anaesthetized with intraperitoneal pentobarbital (5 mg/100 g body weight), exsanguinated through the abdominal aorta, and samples of muscle, liver, and bone obtained. Analyses of these tissues for water, neutral fat, sodium, potassium and chloride were made by methods previously described by the authors(1,3). Plasma was analyzed for water, sodium, potassium, and chloride. In addition, total phosphate was measured in muscle by methods previously reported(1,4) and calcium in bone by a modification of Bergstrom's method(5).

The size of the extracellular space in muscle and bone was estimated using the assumption that all the chloride in the tissue sample was extracellular(6). Plasma ultrafiltrate concentrations of electrolytes were calculated from their concentrations in plasma water, using a Donnan distribution factor of 0.96(7). Bone mineral, used as the reference base for bone water and electrolytes, was assumed to constitute two-thirds of the fat-free dry solids of both control and experimental animals(8).

Analyses of the liver in the rat yield sodium:chloride ratios which are smaller than those of plasma, indicating that some or all of the cells in rat liver contain chloride (7). Because of this, the distribution of chloride cannot be used to measure the size of the extra-cellular space in this tissue. Accordingly, the following calculation was devised in order to estimate this space in rat liver. It was assumed that 1) intracellular sodium exists at the same concentration in the cells of liver as in muscle, and 2) that the intracellular water of rat liver approximates 500 cc/kg of fat-free liver(4). The amount of sodium present in this intracellular water was then subtracted from the total amount of sodium found in the tissue, and a "sodium space" calculated from the remainder of the sodium. Such a calculation, while obviously not exact, gives values for intracellular liver potassium concentration which closely approximate those found in the muscles and red cells of the rat(15) and values obtained in rabbit liver using a chloride space(4).

TABLE I. Plasma Composition in Control and Sodium-loaded Rats.

	pH	H_2O, g/l	Na	K	Cl
			⌐ meq/l plasma ¬		
Control	7.53	944	140	3.7	105
	7.48	943	144	4.3	105
	7.50	948	141	3.9	104
Avg	7.50	945	142	4.0	105
Na-loaded	7.57	949	140	3.8	104
	7.51	956	140	3.6	105
	7.41	950	140	4.0	106
	7.48	951	139	3.7	100
	7.49	954	140	3.8	102
Avg	7.49	952	140	3.8	104
p	>.5	>.10	>.10	>.5	>.5

Statistical comparisons of the data from the 2 groups of animals were made using Student's t-test, incorporating Bessel's correction for small samples.

Results. The control rats had an average weight of 385 g at the beginning of the experiment and 394 at the end. The average weight of the sodium-loaded rats rose from 405 to 409 g. There was no significant difference between the 2 groups, and no excessive weight gain in the experimental animals.

Tables I and II present the values obtained on analysis of plasma, muscle, liver, and bone in the 2 groups of animals. Despite the heavy sodium load, plasma composition remained unchanged in the experimental animals, with the exception of a slight statistically insignificant increase in the plasma water.

TABLE II. Tissue Composition in Control and Sodium-loaded Rats.

	H_2O, g	Na, meq	K, meq	Cl, meq	PO_4, mM	Ca, mM
	(units/100 g fat-free dry solids)					
Control muscle	310	7.9	46.9	4.8	31.6	
	313	7.5	46.0	4.8	30.6	
	315	8.5	46.4	4.8	28.5	
Avg	313	8.0	46.4	4.8	30.2	
Muscle Na-loaded	331	8.4	45.6	5.2	29.4	
	314	7.9	46.2	5.2	29.3	
	322	6.8	48.7	4.9	30.4	
	317	7.6	44.0	5.1	29.8	
	326	9.5	45.8	4.9	29.7	
Avg	322	8.0	46.1	5.1	29.7	
Control liver	242	8.8	31.1	9.0	36.4	
	233	8.3	33.7	9.4	34.4	
	245	8.4	33.0	8.6	37.8	
Avg	240	8.5	32.6	9.0	36.2	
Liver Na-loaded	257	8.5	33.4	10.3	38.4	
	237	7.6	34.1	9.2	36.1	
	245	8.5	36.9	9.8	38.1	
	234	7.7	32.3	9.1	34.9	
	257	7.7	35.4	10.4	40.0	
Avg	246	8.1	34.5	9.7	37.5	
Control bone	33.2	30.4	1.9	2.75		651.2
	27.7	30.5	1.7	2.30		707.5
	29.1	30.2	2.0	2.44		700.0
Avg	30.0	30.4	1.9	2.50		686.2
Bone Na-loaded	25.3	32.7	1.7	2.41		663.0
	24.4	32.0	1.6	2.25		671.5
	21.2	31.2	.9	2.10		687.0
	21.3	33.0	1.0	2.04		680.0
	21.5	32.0	1.0	2.18		
Avg	22.7	32.2	1.2	2.19		675.0

In the soft tissues, muscle and liver, the basic data also show little change, with the exception of a slight increase in the water and chloride content of the experimental tissues. Potassium and phosphate in muscle both decline, while in liver, both increase. Neither change is striking.

Bone, in contrast to muscle and liver, shows a decrease in water of 7.3 g/100 g of dry fat-free solids, equivalent to a loss of 24% of total bone water. There was an increase of 1.8 meq of sodium/100 g of fat-free dry solids, or 6%. Potassium, chloride, and calcium concentrations fell slightly.

Discussion. Table III presents the extracellular spaces and the intracellular ion concentrations found in muscle and liver. The changes observed in these tissues in response to sodium loading were small and tended to be in opposite directions in the 2 tissues. The ECF of muscle increased 5% in the sodium-loaded rats, while liver ECF decreased 4%. Intracellular muscle potassium concentration decreased slightly, while liver potassium increased an equally small amount. Using the sodium space in liver as a measure of the extracellular fluid, as described, values for intracellular chloride of 15-21 meq/kg of ICW were found. The values were somewhat higher in the experimental animals. This chloride may be distributed uniformly through the liver cells or, more probably, may be present at a higher concentration in the Kupfer cells(7). The exact location cannot be determined from the data presented here. There was no increase in the intracellular sodium of muscle. Actually, it was somewhat lower than in the control animals. Since an assumed value for intracellular sodium was used in liver, based on muscle composition, no data are available as to its actual intracellular concentrations. However, there was no increase in the total amount of liver sodium (Table II). None of the changes observed in these tissues were statistically significant. The small reciprocal shifts in ions observed in liver and muscle are in keeping with the findings of other workers concerning the respective *in vitro* ion requirements of these tissues for optimum carbohydrate metabolism (9,10). Opposite responses of the intracellular electrolytes of liver and muscle have also been described by the authors in acidosis in dogs(1).

TABLE III. Average Intracellular Composition of Muscle and Liver in Control and Sodium-loaded Rats.

	Extracellular space, cc/kg tissue H_2O	Na	K	Cl	P
		mMols/kg intracellular H_2O			
Control musc.	133	7.2	171	(0)*	112
Muscle Na-loaded	139	6.2	166	(0)*	108
p	>.5	>.5	>.5		>.5
Control liver	207	(7.2)†	170	15.3	190
Liver Na-loaded	198	(6.2)†	174	20.3	190
p	>.5	>.5	>.5	>.5	>.5

* Assumed not to penetrate cell membrane.
† Assumed on basis of muscle composition.

TABLE IV. Average Changes in Bone Water in Chronic Sodium Loading.

	Total H_2O	Extracellular H_2O	Intracellular H_2O + H_2O of crystallization
	g/kg bone mineral		
Control	450	324	126
Na-loaded	340	291	50
% change	−24	−10.2	−60
p	<.05	>.1	<.01

TABLE V. Average Changes in Bone Electrolytes in Chronic Na Loading.

	Na	K	Ca
	meq/kg bone mineral*		
Control	408	26.6	10,301
Na-loaded	443	17.6	10,145
% change	+8.6	−34.0	−1.5
p	<.02	>.1	>.1

* Corrected for ECF electrolytes.

In contrast to the constancy of liver and muscle composition following sodium loading, bone exhibited considerable change in both sodium and water content. These changes are presented in Tables IV and V. Values are presented as units/kg bone mineral, and, in the case of the electrolytes, have been corrected for the electrolyte contained in the extracellular fluid. This base of reference was employed because approximately one-third of the total bone solids are organic collagen(11), the water of which is more than 90% extracellular in composition(3). When the electrolytes present in the ECF of bone are subtracted from total bone electrolytes, the organic matrix is virtually electrolyte-free, the remainder of the ions being associated with the crystalline solids. *In vivo,* sodium is probably limited to the surface area of these crystals, or to two-thirds of the total mineral(8), so that its effective concentration in the skeleton is probably higher than the figures presented here.

Table IV presents changes in the water content of bone occurring in response to sodium loading. A significant loss of 24% of total bone water occurred in the experimental animals. There was a small loss of extracellular water, but the major part of the water loss was accounted for by a decrease of 60% in the sum of the intracellular and crystal water of the bone. In the dense cortical bone studied here, cell water probably constitutes only some 5% of total bone water; therefore, it is probable that most of this water was lost from the crystalline phase of bone water, due to rearrangement or loss of the hydration shells of the crystals.

Since the extracellular spaces in the bones of the experimental animals were smaller than in the controls, it is apparent that no sodium was added to bone from this source. Sodium associated with bone mineral showed a rise in concentration from 408 meq in the control bones to 443 meq in the experimental bones, representing an 8.6% increase in bone crystal sodium. Changes in potassium and calcium were not statistically significant.

A reciprocal relationship between water and sodium concentrations in bone mineral has been described by the authors in acute sodium depletion due to mercurial diuretics and also in response to aging(12). The equimolar, heteroionic exchange of sodium for hydronium ions has been demonstrated in bone(8), and such a simple exchange may account for part of the water lost in these animals. Sodium can also substitute for calcium at the surface of the bone crystals, on an equimolar basis (13), and a small, though statistically insignificant, fall in bone calcium was noted in the experimental bones. If such an exchange did occur, it would involve the substitution of a univalent ion for a divalent ion, and, furthermore, an ion whose ionic radius in the hydrated state is smaller than calcium. Both of these factors could effect a reduction of the hydration shell of the crystal, resulting in a decrease in bone water. Whether either or both of these mechanisms plays a role in the changes in sodium and water concentrations reported here is purely speculative.

The relative constancy of the plasma, muscle and liver composition in these animals, despite the massive loads of sodium chloride, is in keeping with the plasma data of Gamble *et al.* in infants given large loads of sodium salts(14) and with the data of the authors on the cellular tissues of dogs and rats during various forms of sodium depletion(1,12). The failure of muscle and liver to show an in-

creased sodium content after sodium loading confirms the impression gained from these previous studies; namely, that the sodium content of extracellular fluid and soft tissue cells is closely guarded in the normal animal and can only be altered by extreme situations. The changes in the sodium content of bone mineral, on the other hand, confirm the concept that this phase of the skeleton acts as a reservoir of sodium which is able, under appropriate circumstances not only to release sodium into the extracellular fluid, but also to store this ion.

Summary. 1) The composition of plasma, muscle, liver and bone was examined in adult male rats. Three rats served as controls, and 5 rats were given a daily load of sodium chloride approximating 50% of the total body sodium for 24 days. 2) The sodium content of plasma, muscle and liver was unchanged in the experimental animals at the end of this time. However, bone mineral sodium increased significantly, and bone water declined.

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Effect of Fasting or Underfeeding on Glucose Tolerance of Rats.* (23625)

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Humans(1) and rabbits(2) fasted for several days show glucose tolerance curves of the diabetic type, *i.e.,* increased elevation and delayed decline of blood sugar levels after glucose administration. The degree of diminution in glucose tolerance is proportionate to the duration of the fast. Hill *et al.*(3) found that in rats fasted for 96 hours (sex, weight and previous diet not specified) which received 400 mg glucose/100 g body wt by stomach tube, blood sugar progressively rose and reached 280 mg% 5 hours after the gavage, whereas it did not exceed 130 mg% if the rats were fed a diet rich in glucose during the 96 hours preceding the tolerance test. We have investigated more extensively the glucose tolerance of rats fasted for 1 to 8 days and rats subjected to chronic undernutrition, which causes a variety of changes in carbohydrate metabolism(4,5,6).

Method. Male Sprague-Dawley rats fed Rockland diet were used. Glucose was given by gavage (300 mg/100 g body wt., 30% solution, or 400 mg/100 g, 40% solution) or into the saphenous vein exposed without anesthesia (100 mg/100 g, 40% solution). Oral glucose tolerance tests were performed in rats fasted for 1, 4, 6 or 8 days and intravenous tolerance tests also in rats which were not fasted. Before fasting these animals were fed

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† Medical student summer fellow.

TABLE I. Duration of Fast and Oral Glucose Tolerance of Previously *Ad libitum*-Fed Rats.

Days fasted	No. rats	Body wt, g	Initial blood sugar, mg %	Blood sugar change, mg %* Hours after glucose ½	1	2	3	4	Dose glucose, mg/100 g
1	8	217 ± 6†	73 ± 3	+39 ± 7	+39 ± 7	+35 ± 4	+27 ± 5		300
4	8	185 ± 3	77 ± 2	+41 ± 3	+51 ± 5	+38 ± 4	+30 ± 5		"
6	8	166 ± 4	76 ± 2	+28 ± 4	+32 ± 5	+23 ± 3	+22 ± 2		"
8	4	147 ± 2	62 ± 3	+44 ± 4	+70 ± 4	+69 ± 9	+48 ± 9		"
4	8	253 ± 5	70 ± 1	+33 ± 5	+31 ± 4	+47 ± 4	+52 ± 5		400
4	8	265 ± 5	82 ± 2		+22 ± 5	+23 ± 6	+38 ± 6	+42 ± 7	"

* Compared with initial value. † Mean ± stand. error.

ad libitum. Further rats (230-250 g) were placed for 6-7 weeks on a regimen of 10 g ground Rockland diet daily and water *ad libitum*. Controls fed the same diet without restriction consumed an average of 20 g daily. They were either of the same age as the experimental rats (age controls) or selected to match their final weight (size controls). Experimental and control animals were fasted for 24 or 96 hours before the administration of glucose by stomach tube (300 mg/100 g, 30% solution) or intravenous injection (100 mg/100 g, 40% solution). Blood was taken from the tail vein before and at intervals (*cf.* Tables) after the feeding or injection of glucose. Blood sugar levels were determined as in earlier studies(5). Liver glycogen levels were measured in groups of underfed rats and size controls which were tube-fed glucose (300 mg/100 g) after a 22 hour fast and killed 2 hours later, and in animals which had received no glucose during the terminal 24-hour fast. Under Nembutal anesthesia (5 mg/100 g) liver pieces weighing about 1 g were removed, frozen between blocks of dry ice and weighed in the frozen state. Dissolved alcoholic precipitates of 30% KOH digests were then analyzed with the method of Montgomery(7). In some underfed rats the penis was ligated and bladder urine accumulated in the course of the oral glucose tolerance test examined for sugar with Benedict's reagent.

Results. Table I shows that prolongation of acute fasting up to 6 days failed to result in the progressively diminishing oral glucose tolerance described in rabbits(2). Rats fasted for 8 days were moribund (9 out of 13 had died between the 6th and 8th day of starvation) and their decreased glucose tolerance could therefore have been due to nonspecific changes such as impaired circulation. Even rats given 400 instead of 300 mg/100 g of glucose after a 4 day fast did not show the exorbitant elevation of blood sugar described by Hill *et al.*(3) in similarly treated animals. Reasons for this discrepancy are not obvious, perhaps owing to lack of particulars in Hill *et al.*'s paper. In' intravenous glucose tolerance tests non-fasted animals showed a faster return of blood sugar levels towards normal than fasted rats, but a significant change in slope of the blood sugar decline did not occur as fasting time was prolonged from 1 to 6 days (Table II). The 3 rats fasted for 8 days (survivors of 19 still alive at 6 days) showed a markedly reduced rate of removal of excess glucose from blood, but because of the poor condition of these animals the significance of

TABLE II. Duration of Fast and Intravenous Glucose Tolerance of Previously *Ad libitum*-Fed Rats.

Days fasted	No. rats	Body wt, g	Initial blood sugar, mg %	Blood sugar change, mg %* Min. after glucose 10	30	60
0	6	257 ± 2†	101 ± 3	+ 90 ± 6	+ 36 ± 9	+15 ± 3
1	6	234 ± 3	72 ± 1	+121 ± 4	+ 81 ± 5	+38 ± 4
4	8	190 ± 2	82 ± 1	+ 92 ± 2	+ 73 ± 4	+36 ± 4
6	8	126 ± 2	79 ± 3	+ 90 ± 3	+ 68 ± 6	+31 ± 8
8	3	151 ± 1	52 ± 8	+ 95 ± 8	+108 ± 7	+89 ± 12

* Compared with initial value. † Mean ± stand. error.

TABLE III. Oral Glucose Tolerance of Underfed and Control Rats.

Regimen	Days fasted	No. rats	Body wt, g	Initial blood sugar, mg %	Blood sugar change, mg %* Hours after glucose ½	1	2	3
Underfed	1	15	208 ± 5†	82 ± 1	+37 ± 3	+40 ± 3	+15 ± 3	+ 2 ± 5
Ad lib fed‡	1	8	210 ± 1	69 ± 3	+58 ± 4	+48 ± 4	+47 ± 2	+27 ± 5
Idem §	1	6	350 ± 4	67 ± 3	+24 ± 3	+30 ± 3	+30 ± 5	+23 ± 4
Underfed	4	7	182 ± 2	78 ± 6	+37 ± 8	+29 ± 4	+32 ± 3	+32 ± 3
Ad lib fed‡	4	8	185 ± 3	77 ± 2	+41 ± 3	+51 ± 5	+38 ± 4	+30 ± 5

* Compared with initial value. † Mean ± stand. error. ‡ Size controls. § Age controls.

this change is in doubt.

In the oral glucose tolerance test the blood sugar levels of underfed rats fasted for 1 day declined faster than those of controls. Age controls showed a lower blood sugar zenith than size controls, which agrees with earlier findings on the effect of body weight on glucose tolerance(8). Underfed rats fasted for 4 days showed only a somewhat flatter glucose tolerance curve than controls (Table III). In the intravenous glucose tolerance test (Table IV) underfed rats fasted for 1 day again exhibited the fastest return to preinjection blood sugar levels, but the age controls did not lag far behind, whereas in size controls the blood sugar fall was definitely more sluggish.

Previous studies(5) had shown that intestinal absorption of glucose is not impaired in underfed-fasted rats. Since we had also found(5) that liver glycogen levels of underfed rats change only slightly when a test meal is fed after a 1-day fast, the possibility was considered that the fleeting blood sugar rise in underfed rats given glucose orally may be due to failure of hepatic glycogen formation. However, 2 hours after tube-feeding of glucose underfed rats had liver glycogen levels commensurate with those of similarly fed size controls (Table V). As in previous studies(5), the fasting liver glycogen concentrations were significantly ($p<.01$) higher in underfed rats than in controls. Glycosuria was not found in any of the underfed-fasted rats tested.

The tendency of underfed rats fasted for 1 day towards increased glucose tolerance (especially in the oral test) may be related to their enhanced insulin sensitivity(5). The faster fall of blood sugar levels in these animals is conceivably due to the exaggerated response to insulin secreted under the stimu-

TABLE IV. Intravenous Glucose Tolerance of Underfed and Control Rats.

Regimen	Days fasted	No. rats	Body wt, g	Initial blood sugar, mg %	Blood sugar change, mg %* Min. after glucose 10	30	60
Underfed	1	8	203 ± 2†	90 ± 4	+ 88 ± 4	+44 ± 5	− 2 ± 2
Ad lib fed‡	1	8	199 ± 1	70 ± 2	+100 ± 3	+79 ± 5	+33 ± 6
Idem §	1	7	332 ± 5	84 ± 2	+107 ± 3	+48 ± 4	+13 ± 2

* Compared with initial value. † Mean ± stand. error. ‡ Size controls. § Age controls.

TABLE V. Effect of Fed Glucose on Liver Glycogen of Underfed–fasted and *Ad libitum* Fed–fasted Rats.

	No glucose			2 hr after glucose		
	No. rats	Body wt, g	Liver glycogen, mg %	No. rats	Body wt, g	Liver glycogen, mg %
Underfed–fasted	11	191 ± 2*	479 ± 70	12	194 ± 3	1276 ± 142
Ad lib fed–fasted	11	199 ± 1	145 ± 7	11	200 ± 1	1190 ± 53

* Mean ± stand. error.

lus of hyperglycemia.

Conclusions. Rats fasted for 1-6 days failed to show a progressive decrease of oral glucose tolerance. Return of blood sugar levels to normal after intravenous glucose was somewhat delayed after 1-6 days of fasting as compared with the fed state, but not proportionately with the duration of fast. Prolonged acute fasting therefore did not lead to typical "starvation diabetes" in rats. Chronically underfed rats fasted for 1 day and given glucose orally or intravenously exhibited a more fleeting elevation of blood sugar than *ad libitum* fed-fasted controls. This was not associated with any marked deficiency in hepatic glycogen formation from fed glucose, nor with glycosuria.

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Blood Chemistry and Parietal Eye of *Anolis carolinensis.** (23626)

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Although the parietal eye of reptiles has received considerable histological study (Tilney and Warren(8); Von Haffner(9)) its possible function remains unclear. In the present study, quantitative determinations of various organic components of the blood of *Anolis carolinensis* were made to ascertain if removal of the parietal eye affected these components. Previous studies concerning possible functions of this structure have indicated that surgical removal of the parietal eye of adult *Anolis carolinensis* caused testicular stimulation (Clausen and Poris(3)) and modification of the rate of oxygen consumption (Clausen and Mofshin(2)).

Materials and methods. The lizards (*Anolis carolinensis*) were collected in New Orleans several days prior to use. Four experiments, each involving 3 groups, were performed between February and April. Two of these experiments were of 26 hours duration and 2 were of 18 days duration. The parietal eye was removed by thermal cauterization of an etherized lizard. A drop of collodion was used to seal the wound in operated and sham-operated lizards. The sham-operation was performed by cauterization of a small midline area on the surface of the head at the level of the anterior border of the lateral eyes. Only lizards in 18-day experiments were supplied with meal worms (larvae of *Tenebrio molitor*) daily except on the last day. Droplets of water were sprinkled on the cage and on dead vegetation within the cage. At the end of each experiment, the blood of each group was analyzed. Blood was obtained by decapitation; potassium oxalate was used as the anticoagulant. Determination of uric acid was according to the method of Folin(7). Other determinations followed the technics put forth in the *Manual of Standardized Procedures for Spectrophotometric Chemistry,* edited by Fister(6). Accordingly, the lipoid-phosphorus method was basically that of Fiske and Subbarow, the urea method that of Gentzkow and Masen. The determinations of glucose, total nitrogen and non-protein nitrogen were based upon the methods and

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† The work was equally divided and neither person is to be considered as the senior author.

TABLE I. Blood Chemistry of *Anolis carolinensis* after 26 Hour Parietal Eye-Ectomization.

	Intact	Sham-operated	Operated		Intact	Sham-operated	Operated
	6♂, 12♀	4♂, 16♀	4♂, 16♀		6♂, 4♀	6♂, 4♀	6♂, 4♀
Ending 3/22/57	1.8 g*	1.9 g	1.9 g	Ending 3/27/57	3.2 g	3.1 g	3.2 g
Non-protein nitrogen, mg %	73.9 (2)†	63.0 (2)	76.9 (3)	Glucose, mg %	226 ± 11‡ (4)	192 ± 11 (4)	179 ± 10 (4)
Glucose, mg %	170 (1)	197 (2)	124 (2)	Uric acid, mg %	5.80 ± .21 (3)	6.10 ± .25 (4)	6.58 ± .21 (3)
Uric acid, mg %	6.95 (1)	5.15 (2)	3.40 (1)				

* Mean wt of group. † No. of determinations. ‡ Mean followed by stand. error of mean.

modifications of Folin and Wu. The determinations were made on the pooled blood of each group. This was necessary due to the small amount of blood obtainable from each animal (0.05 to 0.2 ml). All lizard heads were fixed in Bouin's fluid. Selected heads were embedded in tissuemat and sections studied histologically. Bodian's(1) protargol method was used for parietal eye "nerve fibers." All testes of lizards in the second 18-day experiment were weighed after fixation. A testis from each experimental group was studied histologically.

Results. Tables I and II set forth the results of the 4 experiments. In most instances the values obtained are similar to the normal values obtained of *Anolis carolinensis* by Dessauer(4,5). The data of the first 26-hour experiment, while insufficient to be analyzed statistically, suggested that removal of the parietal eye 26 hours earlier reduced the blood level of glucose and uric acid. These changes were not confirmed when the experiment was repeated. There are no statistically significant differences between the sham-operated and the operated groups in the second 26-hour experiment. In the experiments in which the parietal eye had been removed 18 days earlier no statistically significant difference between sham-operated and operated lizards in either total nitrogen, non-protein nitrogen or glucose were found. However, significant differences in uric acid, urea and lipoid-phosphorus were detected between sham-operated and operated lizards. These alterations in blood-chemistry can be interpreted as a reflection of the weight and age differences of the experimental groups.

Histological examination of serial sections of the 9 lizard heads in the first 18-day experiment in which the parietal eye was removed, indicated that the parietal eye was completely destroyed in all cases. No obvious differences were noted in the color or locomotor ability of parietal eyeless lizards and their controls. Sham-operated and parietal eye-ectomized lizard groups ate less during the first part of the 18-day experiments, but these groups were

TABLE II. Blood Chemistry of *Anolis carolinensis* after 18 Day Parietal Eye-Ectomization.

	Intact	Sham-operated	Operated
Ending 3/9/57	5♂, 2♀ 5.9 g*	5♂, 3♀ 4.5 g	5♂, 4♀ 4.8 g
Total nitrogen, %	2.45 ± .16†(4)‡	2.70 ± .14 (3)	2.44 ± .04 (3)
Non-protein, nitrogen, mg %	—	71.6 ± 1.1 (3)	84.7 ± 5.4 (4)
	Intact	**Sham-operated**	**Operated**
Ending 4/6/57	20♂, 1♀ 5.6 g	15♂ 3.9 g	16♂ 5.0 g
Non-protein nitrogen, mg %	63.8 ± 1.1 (3)	—	—
Glucose, mg %	203 ± 4 (10)	188 ± 10 (7)	200.4 ± 4.0 (9)
Uric acid, mg %	3.78 ± .07 (9)	5.19 ± .25 (8)	3.57 ± .17 (9)
Urea, mg %	5.80 ± .52 (9)	3.36 ± .31 (8)	4.32 ± .37 (10)
Lipoid-P, mg %	21.4 ± .8 (9)	15.5 ± .2 (7)	19.3 ± .4 (8)

* Mean wt of group. † Mean followed by stand. error of mean. ‡ No. of determinations.

eating as much as the intact animals toward the end of the experiments.

Mean testicular weights for lizards in the 18-day experiment were 47 mg (intact), 33 mg (sham-operated), and 43 mg (parietal eyeless). The lesser testicular weight of the sham-operated lizards may likely be explained by the lesser mean body weight of the group. Histological examination of a testis in each group showed very similar pictures of seasonal stimulation; the seminiferous tubules were well developed and spermatozoa were abundant in each testis.

Several parietal eyes were studied histologically to ascertain the condition of lens, retina, and the presence or absence of parietal eye nerve. In the central region of the lens, deposition of pigment in the cytoplasm of the lens cells was found. In the retina, circumscribed areas of lens-like cells were found where normal differentiation of retinal elements did not take place. Examination of 4 μ sections clearly revealed that the sensory cells (rods) do not contain melanin granules. The melanin pigment of the retina is contained within separate pigment cells. These pigment cells appear to be completely filled with melanin granules. This agrees with the work by von Haffner(9) on *Lacerta vivipara*. An abundantly nucleated, "nerve-like" structure attaches to the connective tissue capsule of the parietal eye and runs caudally into the region of the epiphysis. Study of serial sections failed to reveal a convincing connection of this "nerve-like" structure to the retinal elements, on the one hand, or to the brain in the region of the habenular ganglia, on the other hand. After staining with protargol, no nerve fibers were seen in this "nerve-like" structure, whereas nerve fibers in the brain were clearly demonstrated.

Summary and conclusions. In the present experiments on *Anolis carolinensis,* no significant quantitative alteration, which could be attributed solely to the complete ablation of the parietal eye, was found in the organic components of the blood which were studied. If the parietal eye influences physiological processes at certain times, it probably must do so by means of a secretion produced by the sensory cells themselves.

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Positive Mucin Clot Test in Supernates of Cultures of Avian Embryonic Brain.* (23627)

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Methods. Tissue cultures in Carrel flasks and in roller tubes were prepared from the lateral walls (corpus striatum) of the hemispheres of the telencephalon of 12- and of 14-day-old chick embryos. The explantation was carried out in a thin plasma layer, and after several hours, when the plasma coagulated, the liquid phase of the culture medium consisting of 20% chick embryo extract, 20% chick amniotic fluid, 30% horse serum, and 30% Earle's balanced salt solution was added.

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Blood vessels were removed from the explants as completely as possible. Renewal of the medium was carried out once or twice a week depending on the state of the cultures. With each feeding the medium to be used as well as the supernates of the cultures were tested for mucin clot formation(8). 0.2 ml of 1 N acetic acid was added to 1 cc of the fluid to be tested. A tight mucin clot, the formation of which could be prevented by testicular hyaluronidase (Wyeth), indicated the presence of highly polymerized hyaluronic acid. By gradual dilution of samples of known hyaluronic acid content, the minimum concentration of hyaluronic acid which gives a positive mucin clot test has been found to be about 30 μg/ml. Fresh undiluted embryo extract prepared in this laboratory gave a mucin clot, the formation of which could be prevented by hyaluronidase. 20% of half diluted embryo extract used in this work contained about 20 μg/ml which is below the minimum concentration for a positive mucin clot test and, in fact, never gave a mucin clot. On the other hand, undiluted chick embryo extract no longer produced a mucin clot after 1 to 2 days incubation at 37°C. Consequently a positive mucin clot test after that time indicates the presence of hyaluronic acid newly produced *in vitro* by fibroblasts. Chick embryo extract may thus be used in any concentration in the culture medium, in which production of highly polymerized hyaluronic acid will be tested by the mucin clot method.

The lowest concentration of hyaluronic acid giving a positive mucin clot test is about 30 μg/ml. Multiplication by 30 of the minimal amount of fluid (equal parts of serum and saline) which prevents mucin clot formation, allowed a rough estimation of the concentration of hyaluronic acid in the original undiluted sample: $X = (1 + y)\ a$, where $y =$ the minimal amount of fluid added to the 1 ml sample for preventing mucin clot formation, and $a =$ the concentration of hyaluronic acid in the diluted sample, assumed to be about 30 μg/ml. The results roughly agree with those obtained by turbidimetric determination† (Table I).

Results. Growth usually started at the second or third day of incubation, when at first spindle-shaped cells and some round cells appeared. Subsequently more or less dense cell mats and sheets consisting of long and slender cells were observed. No neurons could be found. Positive tests for mucin clots in the supernates were not obtained before the third or fourth change of the medium, and at times only after 3-4 weeks. The mucin clot formation could be prevented by addition of 0.10 TRU of hyaluronidase (Wyeth) to 1 ml of the supernate.

Hyaluronic acid produced *in vitro* in different cultures and at varying periods of time was estimated to amount to about 45 to 210 μg/ml depending on the size of the cultures, the growth rate and other unidentified factors. The hyaluronic acid evidently was produced by the outgrowing cells (glia and/or "fibroblasts"), and not by cells inside the explants, since supernates from cultures without outgrowth or with very scarce outgrowth did not give a positive mucin clot test. (Table II).

Discussion. The spaces between the ramifications of axons, myelin, dendrites and neuroglia are not empty. Campbell referred to them as "that terra ignota which remains over when cells, fibers and neuroglia are subtracted"(5). But little has been known about

TABLE I. Order of Magnitude of Hyaluronate Concentration Estimated by Sample Dilution.

Source of hyaluronate		Turbidimetric determination, μg/ml	Sample dilution $x = (1 + y^*)\ 30$, μg/ml
Human synovial fluid	#1	3200	3030
Idem	#2	1520	1230
"	#2 2.5%	40	30
Beef EE_{66}		339	240
Chick EE_{100}		260	240
" EE_{50}		105–120	120
Cultures of rat subcut. tissue		90–140	90
Cultures of chick embryo bone		29–240	75–140

* $y =$ minimal amt in cc of equal parts of saline and horse serum which prevents mucin clot formation, added to 1 cc sample.

† I am grateful to Miss Phyllis Sampson of Dr. Karl Meyer's laboratory for carrying out the turbidimetric determinations.

TABLE II. Positive Mucin Clot Test in Supernate of Cultures of Chick Embryo Brain Tissue.

Tubes	Tubes after 10 days		Tubes after 30 days		Tubes after 47 days	
	Good growth	Mc +	Good growth	Mc +	Good growth	Mc +
10	8	4	7	6	3	3
10	6	5	6	6	4	3
20	11	7	13	9	8	7
12	10	2	8	6	8	8
20	18	0	18	3	18	15

the existence of a ground substance in the central nervous system, and half a century later the presence of a ground substance in the central nervous system has been denied (2). On the other hand, increased spreading (6) in the cerebral cortex was found after treatment with hyaluronidase(3). Recently the presence of mucopolysaccharide between the elements constituting the central nervous system was assumed on the basis of histochemical reactions(4,7). The demonstration by Hess of a positive periodic acid-Schiff (PAS) reaction between the cells of the central nervous system suggested the existence of a ground substance between neuroglia fibrils, axon terminations and dendrites(9). Since hyaluronidase did not alter the red coloration of the PAS reaction, it was assumed that the ground substance consisted of a neutral mucopolysaccharide and not of hyaluronic acid(9). Again more recently, the existence of a substance in the cerebral cortex of the cat which is susceptible to the action of hyaluronidase has been reported(1). The present results show that hyaluronic acid is produced in tissue culture by cells growing from explants of embryonic brain. This constitutes further evidence for the existence in the central nervous system of a hyaluronic acid containing ground substance.

Summary. The supernatant fluid obtained from tissue cultures of avian brain, on addition of 0.2 ml of 1 N acetic acid to 1 ml sample, continued to give for over 2 months a mucin clot the formation of which could be prevented by testicular hyaluronidase. This indicates production of hyaluronic acid in tissue culture by cells, other than neurons, growing from explants of embryonic brain.

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Effect of 5-Nitro and 5-Chloro Benzimidazole Derivatives on Heme Synthesis *in vitro.** (23628)

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We have reported that certain 5-methylbenzimidazole derivatives, particularly 2-ethyl-5-methylbenzimidazole, 2,5 - dimethylbenzimidazole and 5,6 - dimethylbenzimidazole prevented incorporation of N^{15} from N^{15}-glycine into heme by chicken erythrocytes during *in vitro* incubation(1,2). Since the same benzimidazole derivatives also were found to inhibit virus duplication(3), we proposed that they might be inhibiting an important metabolic reaction fundamental to more than one biosynthetic process(1,2). They ap-

pear to affect an early step in glycine utilization since they prevented porphyrin synthesis from glycine but not from *delta*-aminolevulinic acid(4). We also have reported that 2, 5-dimethylbenzimidazole and 2-ethyl-5-methylbenzimidazole prevented production of liver tumors in rats fed azo dyes(5,6). These results have prompted investigation of additional 5-substituted benzimidazoles. Using chicken erythrocytes incubated with N^{15}-glycine as a test system, we have compared the effects on heme synthesis of a series of benzimidazole derivatives having nitro or chloro groups instead of a methyl in the 5 position. Similar studies with 5-hydroxybenzimidazole are also included.

Methods. 5-Nitrobenzimidazole, 2-methyl-5-nitrobenzimidazole and 2-ethyl-5-nitrobenzimidazole‡ were prepared by refluxing 4-nitro-*o*-phenylenediamine with the appropriate acid, according to the general procedure of Wagner and Millett(7). 5-Chlorobenzimidazole, 2-methyl-5-chlorobenzimidazole and 2-ethyl-5-chlorobenzimidazole‡ were prepared similarly from 4-chloro-*o*-phenylenediamine. 5-Hydroxybenzimidazole was kindly supplied by Dr. Karl Folkers of Merck and Co. Incubation of chicken blood and N^{15}-glycine, with and without the test compounds, hemin isolation and N^{15} analyses were carried out as described previously(1,2). Three 20 ml samples of the same blood were incubated at the same time, 2 test samples with a control.

Results. Data are presented in Table I showing N^{15} content of hemin isolated from chicken erythrocytes incubated with N^{15}-glycine (control), compared with hemin from erythrocytes incubated with N^{15}-glycine in the presence of 10 mg of the compound indicated (test). Lower N^{15} content is considered indicative of decreased heme synthesis, since samples of the same blood incubated with N^{15}-glycine under identical conditions, or incubated with non-inhibitory supplementary compounds, yield hemin with the same N^{15} content(8). Exp. 1 and 2 indicate that 2-ethyl-5-nitrobenzimidazole was considerably more inhibitory than 5-nitrobenzimidazole or the 2-methyl-5-nitrobenzimidazole. Similarly, in the 5-chloro series (Exp. 3 and 4), 2-ethyl-5-chlorobenzimidazole was considerably more inhibitory than the 5-chlorobenzimidazole or the 2-methyl-5-chlorobenzimidazole. All were more effective inhibitors than benzimidazole itself at this level (Exp. 7 and 8). It also appears that in each instance the 5-chloro derivative was somewhat more effective than the corresponding nitro analogue. 2-Ethyl-5-chlorobenzimidazole, which had almost complete inhibition at this level, was as effective as 2-ethyl-5-methylbenzimidazole, whereas 2-ethyl-5-nitrobenzimidazole was not (Exp. 5 and 6). Benzimidazole itself had only slight activity compared with the 2-ethyl-5-chloro- or 2-ethyl-5-nitro- derivative at this level, even though on a molar basis its concentration was somewhat greater (Exp. 7 and 8). 5-Hydroxybenzimidazole (Exp. 9) was no more effective than benzimidazole itself, and appeared to be even less inhibitory than benzimidazole at the 20 mg level(1,2).

Discussion. 5-Chloro and 5-nitro benzimidazole derivatives, like the 5-methylbenzimidazoles studied previously(1,2) inhibit incorporation of N^{15} from N^{15}-glycine into heme, whereas benzimidazole itself has comparatively little effect. With these compounds also, the 2-ethyl derivatives were more inhibitory than the 2-methyl. Tamm, *et al.*(9) found that 5-chlorobenzimidazole and 2-methyl-5-chlorobenzimidazole inhibited influenza B virus multiplication, and Schneider (10) noted that 6-nitro-benzimidazole (like benzimidazole and 2-ethyl-5-methylbenzimidazole) inhibited tobacco mosaic virus multiplication in tobacco leaf disk culture. Thus 5-chloro- and 5 (or 6)-nitrobenzimidazole,

* We are indebted to Dr. William T. Ham and the Biophysics Department for use of the mass spectrometer, and gratefully acknowledge the technical assistance of Miss Mary Dodson and Mrs. Carl Greever.

† Medical Student Fellow of Natl. Fn. for Infantile Paralysis, summer 1956.

‡ These apparently have not been described previously. 2-Ethyl-5-chlorobenzimidazole melted 170°-171°; analysed (%): C, 59.8; H, 4.89; N, 15.56 (calculated: C, 59.8; H, 4.98; N, 15.51). 2-Ethyl-5-nitrobenzimidazole softened at 169° and melted 179°-180°; analysed (%): C, 57.2; H, 4.81; N, 21.72 (calculated: C, 56.5; H, 4.71; N, 21.98); analyses by Drs. Weiler and Strauss, Microanalytical Laboratories, Oxford.

TABLE I. Effect of 5-Nitro-, 5-Chloro-, and 5-Hydroxybenzimidazole Derivatives on Incorporation of N^{15} from N^{15}-Glycine into Heme by Erythrocytes of Chicken Blood during Incubation *In Vitro*.

Exp.	N^{15} concentration in hemin* Control	Test	Supplement†
	(atom % excess)		
1	.087	.054	5-nitrobenzimidazole
		.026	2-ethyl-5-nitrobenzimidazole
2	.073	.049	2-methyl-5-nitrobenzimidazole
		.020	2-ethyl-5-nitrobenzimidazole
3	.073	.029	5-chlorobenzimidazole
		.008	2-ethyl-5-chlorobenzimidazole
4	.074	.040	2-methyl-5-chlorobenzimidazole
		.010	2-ethyl-5-chlorobenzimidazole
5	.074	.009	2-ethyl-5-methylbenzimidazole
		.009	2-ethyl-5-chlorobenzimidazole
6	.096	.018	2-ethyl-5-methylbenzimidazole
		.036	2-ethyl-5-nitrobenzimidazole
7	.086	.079	benzimidazole
		.015	2-ethyl-5-chlorobenzimidazole
8	.067	.065	benzimidazole
		.026	2-ethyl-5-nitrobenzimidazole
9	.074	.072	5-hydroxybenzimidazole (10 mg)
		.062	" (20 ")

* Values on the order of .002 to .006 atom % excess indicate complete inhibition of N^{15} incorporation into heme(1,2).

† 10 mg of the compound to be tested was added to a flask otherwise identical with the control, except in Exp. 9, where 20 mg was also used.

like the 5-methyl derivatives, are inhibitory in two widely different biological systems, hemoglobin synthesis by avian erythrocytes *in vitro* and virus duplication.

Although methyl, chloro or nitro groups in the 5 position gave compounds having inhibitory activity greater than that of unsubstituted benzimidazole, it is of particular interest to note that 5-hydroxy substitution did not. 5-Hydroxybenzimidazole had only slight, if any, inhibitory activity at the 10 mg level, and appeared to have even less than that of benzimidazole at the 20 mg level. 5-Hydroxybenzimidazole occurs naturally as part of the Vit. B_{12}-like molecule of Factor III, isolated by Friederich and Bernhauer from fermented sewage(11). The biological function and significance of Factor III is unknown. One possible explanation of the inhibitory activities of 5-substituted benzimidazoles might be through blocking a reaction dependent upon the functioning of an active component containing a similar but not identical moiety. The inhibitory effect of 2,5-dimethylbenzimidazole was not altered by addition of Vit. B_{12}(1). This substance contains 5,6-dimethylbenzimidazole, a benzimidazole which also inhibited heme synthesis.

The relative ineffectiveness of 5-hydroxybenzimidazole as an inhibitor of glycine incorporation into heme, and its occurrence in the molecule of a complex naturally-occurring substance of unknown function, suggests the possibility that additional work with inhibitory benzimidazoles and Factor III might indicate whether Factor III plays a role in glycine utilization. Although inhibitory benzimidazoles appear to block a step early in the utilization of glycine, since they block porphyrin synthesis from glycine but not from *delta*-aminolevulinic acid(4), the nature of the step involved, and the relationship, if any, of benzimidazoles to the process, remain to be determined.

Summary. 5-Nitro-, 2-methyl-5-nitro-, and 2-ethyl-5-nitrobenzimidazole; 5-chloro-, 2-methyl-5-chloro-, and 2-ethyl-5-chlorobenzimidazole were prepared and their effects on incorporation of N^{15} from N^{15}-glycine into heme by chicken erythrocytes incubated *in vitro* were studied. Similar to the 5-methylbenzimidazole series studied previously, the 2-ethyl-5-nitro- was considerably more inhibitory than the 5-nitro- or 2-methyl-5-nitrobenzimidazole. Likewise in the 5-chloro series, 2-ethyl-5-chloro- was considerably more inhibitory than the 5-chloro- or 2-methyl-5-chlorobenzimidazole. 5-Chloro derivatives appeared to be more inhibitory than the corresponding 5-nitro analogues; 2-ethyl-5-chlorobenzimidazole was as effective as 2-ethyl-5-methylbenzimidazole, whereas 2-ethyl-5-nitrobenzimidazole was not. All were more effective than benzimidazole itself. Although methyl, chloro or nitro groups in the 5 position gave compounds having inhibitory activity greater than that of unsubstituted benzimidazole, 5-hydroxybenzimidazole was comparatively ineffective.

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